Activation of phosphatidylinositol-3-kinase by interleukin-13 in colonic epithelial cells: regulation of nitric oxide and apoptosis

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ACTIVATION OF PHOSPHATIDYLINOSITOL 3-KINASE BY INTERLEUKIN-13 IN COLONIC EPITHELIAL CELLS: REGULATION OF NITRIC OXIDE AND APOPTOSIS

submitted by

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ABSTRACT

The intestinal epithelium serves as a dynamic barrier which, in the course of its normal function, maintains regulated uptake of nutrients and water at the same time as excluding potential pathogens. Colonic epithelial cells participate in normal homeostasis, innate immunity and regulation of acquired immunity. They produce bioactive cytokines and other immunoregulatory molecules, such as nitric oxide, either constitutively or in response to stimuli including other cytokines or microorganisms. Enteropathies, including inflammatory bowel disease (IBD) result in, or perhaps even from, perturbed epithelial function. The pathophysiology, tissue damage and symptomatology of IBD are due to inappropriate or exaggerated immune reactions, such as unregulated production of pro-inflammatory cytokines or inadequate synthesis of anti-inflammatory cytokines.

Using a colonic epithelial cell line, HT-29, the modulation of the production of nitric oxide was examined. These cells were found to produce nitric oxide (NO) with specific combinations of the pro-inflammatory cytokines interleukin (IL)-1α, interferon (IFN)-γ and tumour necrosis factor (TNF)-α. This NO generation was via the induction of the inducible nitric oxide synthase (iNOS) enzyme, since iNOS mRNA and NO was generated after stimulation with cytokines. Modulation of this effect was achieved with the use of pharmacological agents, indicating a complex array of interacting signalling pathways. The T cell-derived anti-inflammatory cytokine, IL-13 significantly reduced this NO generation and iNOS expression.

Specific combinations of the same pro-inflammatory cytokines initiated programmed cell death in HT-29 cells, an effect that IL-13 was also able to inhibit. An investigation into the intracellular signalling pathway utilised by IL-13 to mediate these effects was undertaken. In this study IL-13 is shown to activate phosphatidylinositol 3-kinase (PI 3-kinase) possibly via the recruitment of the insulin receptor substrate (IRS)-1. PI 3-kinase activation was both essential for the down-regulation of iNOS and critical as a survival signal.

IL-13 also activates protein kinase B (PKB) in a PI 3-kinase-dependent manner. This enzyme is thought to provide the anti-apoptotic signal. In addition, IL-13 delayed the cytokine-induced activation of essential apoptotic mediators, caspases 8 and 3.

For Dad

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Table 5.1 Apoptosis

ABBREVIATIONS

Ab	Antibody
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
AP-1	Activator protein-1
APC	Antigen presenting cells
APS	Ammonium persulphate solution
ASA	5-Aminosalysilic acid
ATP	Adenosine triphosphate
BCR	Breakpoint cluster region
BH_4	Tetrahydropterin
BSA	Bovine serum albumin
Ca^{2+}	Calcium ions
cAMP	Cyclic adenosine-5'-monophosphate
Caspases	Cascade of aspartate specific cysteine proteases
CD	Clusters of differentiation
CD	Crohn's disease
cDNA	Complementary deoxyribonucleic acid
Ced genes	*Caenorhabditis elegans* death genes
cGMP	Cyclic guanisine-5'-monophosphate
CHO	Chinese hamster ovary cells
CHX	Cycloheximide
cNOS	Constitutive nitric oxide synthase
COX	Cyclooxygenase
CPM	Counts per minute
CSF	Colony-stimulating factor
CSPD	Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5-chloro) tricyclo [3.3.1.^{3,7}] decan}-4-yl) phenyl phosphate
DAB	3,3' Diaminobenzidine
DAG	Diacyglycerol
DAN	2,3-Diaminonaphthalene
Db-cAMP	Dibutyril cyclic adenosine monophosphate

DED	Death effector domain
DEPC	Diethyl pyrocarbonate
DIG	Digoxigenin
DISC	Death inducing signalling complex
DMEM	Dulbecco's modified Eagles medium
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EBV	Eppstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbant assay
ERK	Extracellular signal regulated kinase
FACS	Fluorescence activated cell sorter
FAD	Flavin dinucleotide
FADD	Fas associated death domain containing protein
FAE	Follicle-associated epithelium
FBS	Foetal bovine serum
FITC	Fluorescein isothiocynate
FLICE	FADD-like ICE
FMLP	N-formyl-methionyl-leucyl-phenylalanine
FMN	Flavin mononucleotide
GALT	Gut-associated lymphoid tissue
GAP	GTPase-activating protein
GDP	Guanosine diphosphate
GM-CSF	Granulocyte/macrophage colony-stimulating factor
Grb	Growth factor receptor binding protein
GTP	Guanosine triphosphate
HBSS	Hanks balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
HLA	Human leukocyte antigen
HLA-DR	D-related HLA
HPLC	High performance liquid chromatography

IBD	Inflammatory bowel disease
ICAM	Intercellular adhesion molecule
ICE	Interleukin-1β converting enzyme
IEL	Intraepithelial lymphocytes
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
iNOS	Inducible nitric oxide synthase
IP-10	IFN-γ-inducible 10
IRS	Insulin receptor substrate
ITIM	Immunoreceptor tyrosine-based inhibition motif
JNK	c-*jun* N-terminal kinase
kDa	Kilodaltons
L-NAA	N^{ω}-amino-L-arginine
L-NMMA	N^{ω}-monomethyl-L-arginine
L-NNA	N^{ω}-nitro-L-arginine
LP	Lamina propria
LPS	Lipopolysaccharide
LY294002	2-(4-morpholinyl)-8-phenyl-4H-1-benzyopyran-4-one
mAb	Monoclonal antibody
MAP kinase	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MLN	Mesenteric lymph nodes
MOPS	3-[N-morpholino]propane-sulphonic acid
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinocleotide
NADPH	Nicotinamide adenine dinocleotide phosphate
NF-κB	Nuclear factor- κB
NGFR	Nerve growth factor
NO	Nitric oxide
NOS	Nitric oxide synthase(s)
NSAID	Non-steroid anti-inflammatory drug

OD	Optical density
OPD	O-phenylenediamine dihydrochloride
PAF	Platelet activating factor
p-ANCA	p-antineutrophil cytoplasmic antibodies
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PDGF	Platelet-derived growth factor
PDK	PI (3,4,5) P_3-dependent kinase
PDTC	Pyrrolidinedithiocarbonate
PGE_2	Prostaglandin E_2
PGI_2	Prostacyclin
PI	Phosphatidyl inositol
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PMA	Phorbol-12-myristate-13-acetate
PMSF	Phenylmethylsulphonyl fluoride
PP	Peyer's patches
PS	Phosphatidyl serine
PT	Pertussis toxin
PTK	Protein tyrosine kinase
RANTES	Regulated on activation, normal T cell expressed and secreted
ROM	Reactive oxygen metabolites
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SH	*Src* homology
SHIP	SH2 containing inositol 5'polyphosphatase
SOD	Superoxide dismutase
TBS	Tris buffered saline
TCR	T cell receptor

TEMED	N, N, N', N'-tetramethylethylene diamine
TGFβ	Transforming growth factor-β
Th	T helper cell
TLC	Thin layer chromatography
TNF	Tumour necrosis factor
TNF-RI	Tumour necrosis factor receptor I
TNF-RII	Tumour necrosis factor receptor II
TOR	Target of rapamycin
Tween-20	Polyoxyethylenesorbitan monolaurate
UC	Ulcerative colitis
ZVAD-FMK	Cbz-Val-Ala-Asp-(OMe)-fluoromethyl ketone

Single letter amino acid code:

A	Ala
C	Cys
D	Asp
E	Glu
F	Phe
G	Gly
H	His
I	Ile
K	Lys
L	Leu
M	Met
N	Asn
P	Pro
Q	Gln
R	Arg
S	Ser
T	Thr
V	Val
W	Trp
Y	Tyr

1. INTRODUCTION

1.1 ANATOMY OF THE COLON

Colonic mucosa is the innermost layer of the large bowel wall. It consists of the colonic epithelium, which covers both the absorptive surface and the crypts, and the lamina propria, found immediately beneath the epithelium (see figure 1A). Colonic epithelium is composed of columnar epithelial cells (which represent the main population), mucus secreting goblet cells and endocrine epithelial cells, whilst intraepithelial lymphocytes (IELs) infiltrate throughout the epithelium. The primary function of the intestinal epithelium is the absorption of nutrients, water and electrolytes from the lumen to the body. However, more recently it has been realised that because most of the antigens encountered by the normal immune system gain access to the body via a mucosal surface, colonic epithelium itself is involved in various immunological and inflammatory processes. The gut-associated lymphoid tissues (GALT) comprise the Peyer's patches (PP), mesenteric lymph nodes (MLN) and large numbers of lymphoid cells scattered throughout the lamina propria (LP) and epithelium of the intestine (figure 1B). The PP, with clearly defined T- and B-cell dependent areas, lie in the submucosa separated from the intestinal lumen by a single layer of cuboidal epithelial cells, the follicle-associated epithelium (FAE). In addition to conventional enterocytes, this epithelial layer contains many lymphoid cells of all types, as well as a unique population of specialised epithelial cells (M cells), whose function is the uptake and transport of antigen into the lymphoid tissue. This arrangement facilitates a close interaction between the epithelial and the lymphoid elements that are found in the PP. Draining the Peyer's patches via lymphatics are the mesenteric lymph nodes. (Mowat and Viney, 1997).

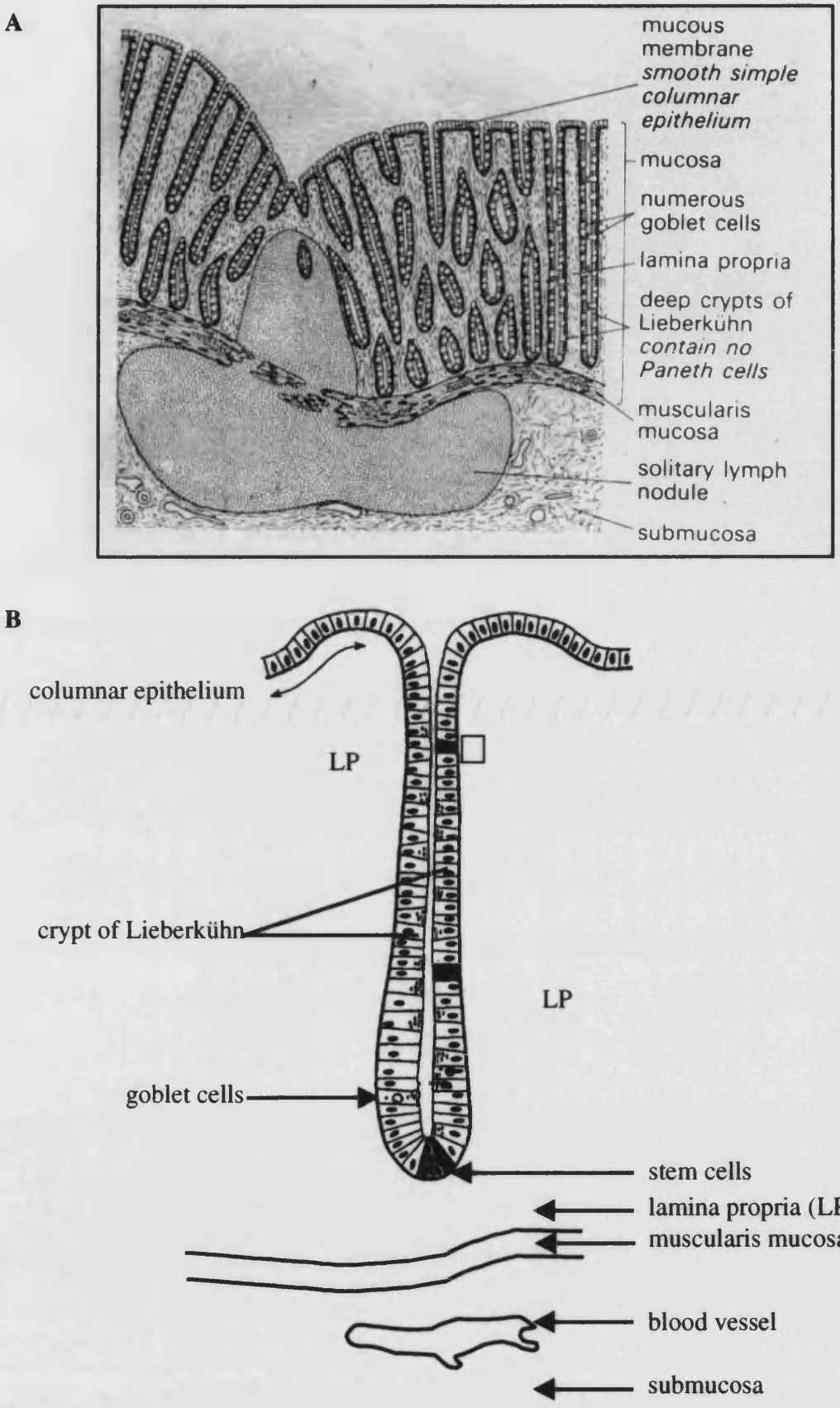


Figure 1.1. Schematic representation of the gut. A. Colon (x40) (adapted from Freeman and Bracegirdle, 1976) and B. Crypts (x140) (adapted from Potten, 1997)

In addition to the organised tissues of the PP and MLN, the crypt units of the colon contain large numbers of scattered lymphocytes, both in the epithelium itself and in the deeper layer of the lamina propria. In contrast to the PP and MLN, which probably act as inductive sites, these scattered LP and IELs play a central role as the effector arm for local intestinal immunity.

1.2 IMMUNOLOGICAL ROLE OF COLONIC EPITHELIUM

The intestinal epithelium represents an important interface between the host and external environment serving both as a surface for absorption and as a defence against ingested pathogens (McKay and Perdue, 1993). Both non-immune and immune mechanisms protect the environment of the lamina propria from challenge by foreign antigens. Intestinal motility, commensal microflora and the mucous coat or glycocalyx comprise some of the non-specific protective barriers. The immune mechanisms can operate within the lumen of the gut, at the mucosal surface or within the lamina propria (Abreu-Martin and Targan, 1996). The intestinal epithelial cells constitute a barrier between the environment and the host tissue and they are the first cells to come in contact with many pathogens. From this position, intestinal epithelial cells might play a crucial role as an outpost of the immune system located in the underlying intestinal mucosa. Soluble mediators produced by these cells might function as an early signal to neighbouring immune cells and be involved in the recruitment of cells during the inflammatory response. Finally, the interplay between the colonic epithelial cells, as "professional" antigen presenting cells (APCs), and IELs may complete the immunological role of colonic mucosa.

The epithelium and lamina propria represent highly distinct compartments of the immune system, despite being separated only by a thin layer of basement membrane. The LP contains most components of the immune system, with large numbers of B cells, plasma cells, macrophages, dendritic cells and T cells of both the $CD4^+$ and $CD8^+$ subsets. Approximately 10 – 15 % of the cells within the normal colonic mucosa are lymphocytes, of which > 90 % are T cells and the majority of those (~80%) are of the $CD8^+$ subset (Mowat and Viney, 1997). IEL are located between the epithelial cells adjacent to the basement membrane and are the only lymphocyte population situated so close to antigen in the entire body.

A role for colonic epithelial cells in inflammatory and immune reactions is increasingly recognised. These cells present antigens via major histocompatibility complex (MHC) Class II molecule expression and act as APCs to T cells (Mayer *et al.*, 1991 and Lowes *et al.*, 1992). Class II MHC determinants are expressed on normal small intestinal epithelial cells but not on colon epithelial cells unless the colon is inflamed (Selby *et al.*, 1983). Expression of Class II antigens may be modulated by IFN-γ produced by activated $CD8^+$ T cells in the epithelium (Cerf-Benussan *et al.*, 1984). The expression of human leukocyte antigen (HLA) molecules by colonic epithelial cells was found to activate $CD3^+/CD8^+$ IELs (Hoang *et al.*, 1992). Colonic epithelial cells express adhesion proteins, such as intercellular adhesion molecule (ICAM)-1 (Kaiserlain *et al.*, 1991 and Kvale and Brandzeig, 1995). In addition, they generate soluble inflammatory mediators. These include arachidonic acid derivatives (Gustafson and Tagesson, 1990 and Dias *et al.*, 1992), platelet activating factor (Ferraris *et al.*, 1993), cytokines (Hedges *et al.*, 1992; Eckmann *et al.*, 1993; Schuerer-Maly *et al.*, 1994; Kolios and Nakos, 1995 and Gross *et al.*, 1995) and chemokines (Mazzucchelli *et al.*, 1994 and Jung *et al.*, 1995), all of which contribute to the communication between inflammatory epithelial cells and lymphocytes of

the immune system (R. B. Sartor, 1994). Colon epithelial cells appear to be programmed to provide a set of signals for the activation of the mucosal inflammatory response in the earliest phases of microbial invasion (Jung *et al.*, 1995). The involvement of colonic epithelial cells in immune and inflammatory reactions in inflammatory bowel disease is discussed in the next section.

1.3 INFLAMMATORY BOWEL DISEASE

The term inflammatory bowel disease (IBD) describes two major pathological conditions involving the gastrointestinal tract, namely ulcerative colitis (UC) and Crohn's disease (CD). In UC the inflammatory disorder is superficial in the mucosa and submucosa and limited to the large bowel, affecting the rectum and a variable extent of the colon. Histologically, the disease is characterised by an infiltration of both acute and chronic inflammatory cells (such as neutrophils, monocytes and plasma cells) in the lamina propria and the crypts, leading to the formation of microabscesses. The deeper layers of the intestinal wall are characteristically not involved. CD is a transmural inflammation that may occur anywhere throughout the alimentary tract from the mouth to the anus. The disease can affect the deeper layers of the bowel wall producing superficial and deep ulceration and also leading to collagen deposition and fibrosis. The cellular infiltration consists of lymphocytes and macrophages and the presence of granulomas is a predominant feature of the disease. Both of these diseases are characterised by chronic relapses and are associated with many extra-intestinal manifestations. (B. E. Sands, 1998).

Despite extensive investigation for decades the aetiology and pathogenesis of both major forms of IBD remains unknown. Increasing evidence suggests that these two disorders are

partly and, possibly, wholly distinct in their initial aetiological event, although they share important common pathogenetic mechanisms. It is likely that the aetiology of IBD is not a single cause and effect relationship and probably represents an interaction between various agents. A number of genetic predisposing factors, exogenous and endogenous triggers and modifying factors is involved and their interaction is an inflammatory process, in which tissue injury seems to be mediated by the immune system (R. P. MacDermott, 1998). The prognosis of ulcerative colitis and Crohn's disease has much improved over the years, but an aetiologic cure has not yet been found, emphasising the need for further investigation of these challenging diseases. Until the cause of these diseases has been clearly identified therapeutic strategies for these conditions must be based on interrupting the immunopathogenetic mechanisms involved and inhibiting the gut inflammation; hence the widespread use of immunosuppressives in their treatment (W. J. Sandborn, 1998 and D'Haens and Rutgeerts, 1998). In the following section, Table 1.1A summarises the most important factors that condition, cause and promote IBD (C. Fiocchi, 1998). Thereafter, a selection of features relevant to this research project will be reviewed in more detail.

Aetiology of IBD

The cause of IBD is likely to be multifactorial. Although the aetiology of IBD remains obscure, a number of genetic, environmental, microbial, and immunological factors responsible for the cause of this disorder have been suggested and multiple aetiological theories have been proposed (Table 1.1B), reviewed in (C. Fiocchi, 1998).

Table 1.1A. Factors thought to Contribute towards the Aetiology of IBD

Component	UC	CD
Environmental factors	Beneficial effect of smoking No beneficial effect of diet Normal intestinal permeability in healthy relatives	Detrimental effect of smoking Symptoms improved by selective diets Increased intestinal permeability in healthy relatives
Genetic associations	Largely different from CD	Largely different from UC
Microbial agents	Limited role of bacterial flora No association with *M. paratuberculosis* No association with measles virus	Important role of bacterial flora Association with *M. paratuberculosis* Some association with measles virus

Table 1.1B. Distinguishing Features of UC and CD Pathogenesis

Component	UC	CD
Humoral immunity	Prominent antibody secretion Evidence of autoimmunity Strong association with ANCA	Moderate antibody secretion Limited evidence for autoimmunity Weak association with ANCA
Cell-mediated immunity	Prominent neutrophil infiltration in the mucosa Normal/hyporeactive T cells Normal T-cell apoptosis (?)	Prominent T-cell infiltration in the mucosa Hyperreactive T cells Resistance of T cells to apoptosis
Cytokines and mediators	Prominent production of eicosanoids Th2-like profile Increased cytokine production limited to involved mucosa	Moderate production of eicosanoids Th1-like profile Increased cytokine production in involved and uninvolved mucosa

ANCA, antineutrophil cytoplasmic antibodies

Pathogenesis of IBD

Although the primary aetiological agents in IBD are still elusive, it is now known that disease activation involves two processes. First, an early increase in expression of surface adhesion molecules on vascular endothelial cells, with consequent diapedesis and activation of circulating leukocytes in gut mucosa (R. P. MacDermott, 1998). Second, an increased synthesis and release of a wide range of inflammatory mediators and cytokines are likely to contribute to the clinicopathological features of IBD (Kolios *et al.*, 1997). The sources of these mediators include not only resident and newly recruited mucosal inflammatory cells such as neutrophils, macrophages and mast cells, but also platelets, colonic epithelial cells, vascular endothelial cells, fibroblasts, smooth muscle cells and enteric neurons (C. Fiocchi, 1998). Figure 1.2 shows the components and events involved in IBD aetiopathogenesis.

Cytokines and IBD

Cytokines are a large family of structurally diverse proteins of 8-30 kDa, which are produced by most nucleated cells, particularly, but not exclusively, immune cells. These proteins are mediators of immunoregulation and inflammation and probably play a key role in intestinal inflammation via the induction and suppression of inflammatory processes. In addition they have a role in the regulation of healing and repair by activating effector cells (Stadnyk and Waterhouse, 1997), by increasing their proliferation and providing chemotactic messages. Individual cytokines modulate the secretion, not only of other cytokines, but also other mediators, such as chemokines. Thus cytokines may be responsible for many of the symptoms experienced by patients with IBD via their local and systemic effects or by inducing the production of other mediators and initiating a cascade of effects (Kolios *et al.*, 1997). Cytokines can be considered as pro-inflammatory (e.g. IL-1, IFN-γ

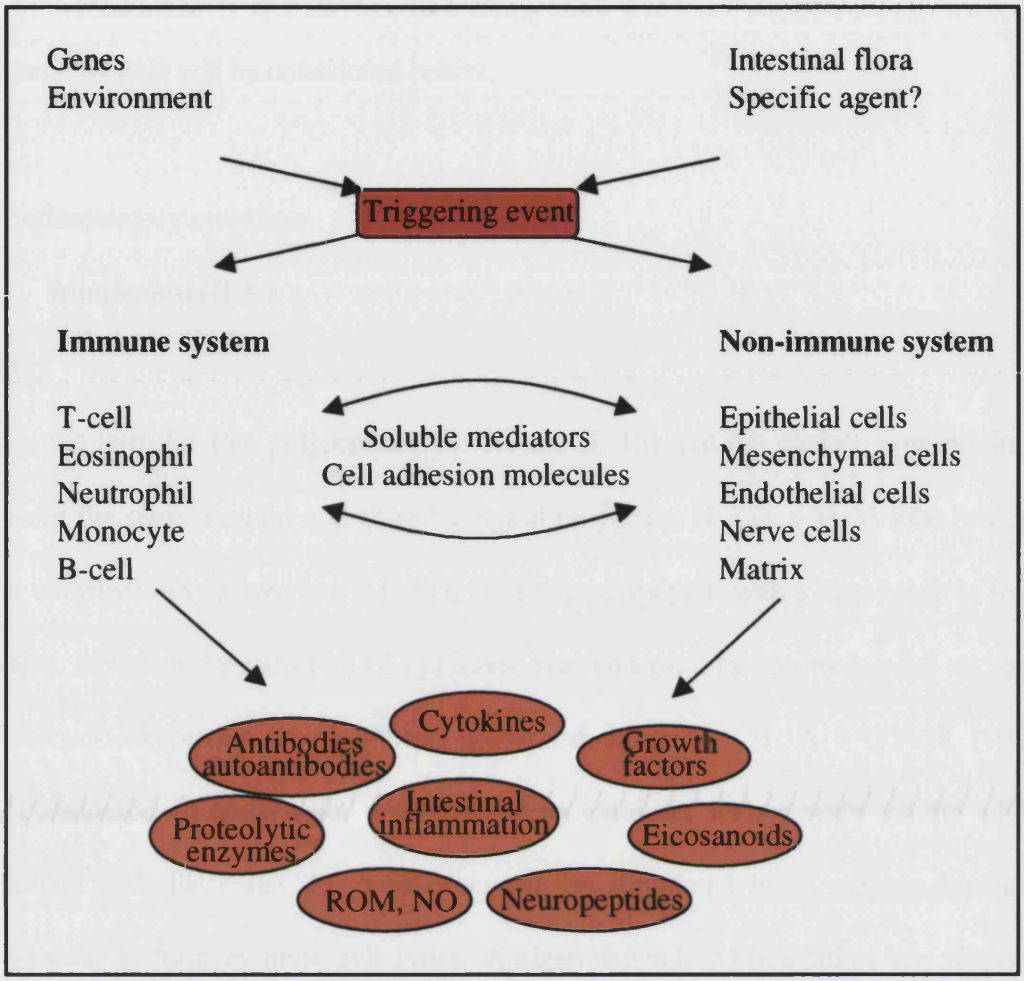


Figure 1.2. Diagram showing components and events involved in IBD aetiopathogenesis. Interacting environmental and genetic factors in combination with the microbial intestinal flora or a still unidentified specific microorganism trigger an event that activates intestinal immune and nonimmune systems. The cell-mediated immune response induces effector T cells and activates macrophages, neutrophils and other leukocytes, whereas the humoral response stimulates B cells to produce antibodies. Through secretion of soluble mediators and expression of cell adhesion molecules, immune and nonimmune cells exchange signals, resulting in further cell activation and amplification of the production of antibodies and autoantibodies, cytokines, growth factors, eicosanoids, neuropeptides, ROMs, NO and proteolytic enzymes culminating in inflammation and tissue damage.

and TNF-α) or anti-inflammatory (e.g. IL-4, IL-13 and IL-10). These cytokines and their relevance to IBD will be considered below.

Pro-inflammatory cytokines

(i) Interleukin (IL)-1

Biology

IL-1 is the term for two polypeptides (IL-1α and IL-1β) that are distinct gene products, but recognise the same receptor and share biological properties. IL-1 is a 31-33 kDa pro-cytokine that is enzymatically cleaved into a 17 kDa bioactive polypeptide with a large range of biological activities, including the induction of cytokines, chemokines, adhesion molecules, and enzymes such as cyclo-oxygenase, soluble phospholipases and collagenases (L. A. J. O'Neill, 1995). It is now considered to be a major pro-inflammatory cytokine, with a wide variety of targets (including epithelial cells). Its production can be stimulated in an antigen-dependent or independent fashion in most cell types. Antigen-dependent stimulation can occur either directly, during antigen presentation when a T cell is in contact with an APC, or indirectly, as a result of the release of cytokines, such as IL-2 or interferon (IFN)-γ, from an activated T cell. Antigen-independent induction of IL-1 can occur when pathogens such as viruses or bacteria, or certain irritants such as asbestos or silica, come into contact with monocytes or macrophages. In addition, other cytokines, such as granulocyte/macrophage (GM)-colony stimulating factor (CSF), transforming growth factor (TGF)-β, tumour necrosis factor (TNF)-α, IFN-α and β, as well as IL-1 itself, can trigger IL-1 release from monocytes or macrophages (C. A. Dinarello, 1996). Some of the consequences of IL-1 production include (i) progression from early to late G_1 in the cell cycle of activated helper and cytotoxic T cells, (ii) induction of prostaglandin synthesis in endothelial and smooth muscle cells, (iii) induction of fever and (iv) initiation of the acute phase response. Thus, IL-1 has potent

pleiotropic effects that can be either local or systemic and is critical in the mediation of host responses to injury and infection (C. A. Dinarello, 1996).

Signalling

In the immune system, the production of IL-1 is typically induced, generally resulting in inflammation. Its effects are mediated by binding to specific receptors, IL-1R Types I and II. IL-1RI signals whereas IL-1RII does not and seems to act as decoy receptor (Stylianou *et al.*, 1992 and Colotta *et al.*, 1993). The IL-1 receptor antagonist (IL-1ra) also binds both receptors and acts a true receptor antagonist. Most of the immune and inflammatory genes induced by IL-1 are nuclear factor (NF)-κB-regulated; making it a key mediator of IL-1 effects in cells (Stylianou *et al.*, 1992).

NF-κB regulates the transcription of genes bearing the κB consensus motif. Transmigration of NF-κB from the cytoplasm to the nucleus is regulated by the IκB family of inhibitory NF-κB-binding proteins. The dissociation of the NF-κB/IκB complex requires both phosphorylation and degradation of IκBs. Numerous stimuli, including IL-1 and TNF-α (Henkel *et al.*, 1993 and Beg *et al.*, 1992) ubiquitinate a kinase that phosphorylates I-κBα at serine residues 32 and 36 on the N-terminus of the molecule (Chen *et al.*, 1996). Phosphorylated IκBα is then selectively ubiquitinated and rapidly degraded via a nonlysosomal, ATP-dependent, 26S proteolytic complex composed of a 700 kDa proteasome (M. Hochstrasser, 1996 and Baeuerle and Baltimore, 1996).

Following TNF-α stimulation, TNF receptor-associated factor-2 (TRAF-2) is recruited to the cytoplasmic portion of the TNF-R1 via the intermediate action of TNF receptor-associated death domain (Hsu *et al.*, 1996). In contrast, IL-1 signals through the action of

IL-1R-associated kinase (IRAK), which associates with IL-1R1 and activates TRAF-6 (Cao *et al.*, 1996). Activated TRAF-2 and TRAF-6 are then able to associate/activate the NF-κB-inducing kinase (NIK) (Malinin *et al.*, 1997). NIK, in turn, associates/activates the IκB kinase (IKK) complex (Stancovski and Baltimore, 1997 and Verma and Stevenson, 1997), which is composed of the IKK-1 and IKK-2 subunits, both critical in mediating cytokine-induced IκB phosphorylation (Mercurio *et al.*, 1997; Woronicz *et al.*, 1997 and Zandi *et al.*, 1997). Activation of the IKK complex leads to specific IκBα phosphorylation/degradation and subsequent release of NF-κB, which migrates to the nucleus and activates transcription of κB-specific genes. TRAF-2 has been shown to play an important role in transducing the TNF-α signal through the IκB/NF-κB axis (Rothe *et al.*, 1995; Liu *et al.*, 1996; Min *et al.*, 1997; Tobin *et al.*, 1998; Song *et al.*, 1997 and Natoli *et al.*, 1997).

Apart from NF-κB, there are other targets for IL-1 signal transduction. Mitogen-activated protein kinases (MAPKs) are involved in a number of cell signalling pathways. These enzymes are proline-directed serine/threonine kinases that are activated by dual phosphorylation of threonine and tyrosine residues, in response to a multitude of extracellular stimuli (Kyriakis and Avruch, 1996). There are at least three distinct MAP kinase signal transduction pathways, each leading to activation of either the extracellular-regulated kinases (ERKs), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) or p38 (Kyriakis and Avruch, 1996). ERK, JNK and p38 are phosphorylated by dual-specificity MAPK kinases (MAPKKs or MEKs), which, in turn, are activated by serine/threonine phosphorylation by MAPK kinase kinase (MEKK). Mammalian ERK1 and ERK2 and their upstream activators MEK1 and MEK2 are acutely stimulated by growth and differentiating factors, e.g., EGF, PDGF and NGF, by receptor tyrosine kinases, heterotrimeric G-protein-coupled receptors, or cytokine receptors. The mammalian JNKs

and p38 are implicated in responses to cellular stress, inflammation and apoptosis. They are activated by lipopolysaccharides, pro-inflammatory cytokines, ionising or ultraviolet radiation, heat shock or hyperosmotic stress (Kyriakis and Avruch, 1996).

IL-1 activates the three major MAP kinase cascades (L. A. J. O'Neill, 1996). IRAK activates JNK1 mediated by TRAF6 (Knop *et al.*, 1998 and Song *et al.*, 1997) and may provide a mechanism for the activation of the transcription factor, AP-1 (Song *et al.*, 1997). In addition, IL-1 activates another kinase termed TNF/IL-1-induced protein (TIP) kinase (Guesdon *et al.*, 1997), although the functional relevance of this is as yet unknown.

Relevance to IBD

Inappropriate or prolonged production of IL-1 has been implicated in the pathogenesis of conditions such as inflammatory bowel disease. In active IBD, isolated peripheral blood mononuclear cells were found to produce increased levels of IL-1 compared to controls (Grottrupwolfers *et al.* 1996), but reverted to control levels in the inactive stages. Significant correlation between the IL-1β production and the activity index of the diseases has been found (Nakamura *et al.* 1992). IL-1β mRNA was found in colonic biopsies from patients with infectious and ischaemic colitis. These results suggest that production of IL-1β is not unique to active IBD but is also increased in intestinal inflammation (Isaacs *et al.* 1992). Organ cultures of involved IBD mucosa spontaneously produced increased amounts of IL-1β compared to normal mucosa (Reimund *et al.* 1996). The increased IL-1 production at the mucosal level in those patients with active IBD is probably derived from the presence of increased number of cells capable of synthesising IL-1 in the inflamed intestinal mucosa (Grottrupwolfers *et al.* 1996). Macrophages have been suggested as the major source of IL-1 in IBD mucosa (Mahida *et al.* 1989), although there may be cell types other than macrophages involved in IL-1 production,

such as T cells and fibroblasts. Finally, levels of IL-1ra were markedly lower in patients with IBD. An imbalance in the ratio of tissue IL-1 and IL-1ra levels in the intestinal mucosa of patients with IBD has been proposed (Cominelli and Pizzaro, 1996).

(ii) Tumour necrosis factor (TNF)-α

Biology

TNF-α (or catchetin) is a representative member of a large family of cytokines that exert a fundamental effect on cell proliferation and death, inflammation and immunological and neuronal cell function (Orlinick and Chao, 1998). TNF-α is a non-glycosylated polypeptide that exists as either a 26 kDa transmembrane protein or as an 85 kDa soluble protein. TNF-α is produced by activated monocytes/macrophages and T-cells and inhibits the growth of certain tumour cells, although the alteration of growth, differentiation and metabolism of other cells has also been attributed to TNF, particularly during development, hematopoeisis and reproduction (Aggarwal and Natarajan, 1997). That this protein is capable of producing such a wide variety of effects is attributable to, (i), the ubiquity of its receptor, (ii), its ability to activate multiple signal transduction pathways and (iii), its ability to induce or suppress the expression of a vast number of genes; including those for growth factors and cytokines, transcription factors, receptors, inflammatory mediators and acute phase proteins (Aggarwal and Natarajan, 1997).

Signalling

TNF-α assembles into homotrimeric complexes in its biosynthesis (Jones *et al.*, 1989). The transmembrane form is bioactive and can bind to its cellular receptors (Perez *et al.*, 1990). A non-cleavable form of TNF-α serves as a superior ligand relative to soluble TNF-α (Grell *et al.*, 1995). TNF ligand family members include TNF-α and FasL. Distinct properties of

these ligands include their orientation in the membrane (their type II topology) and their cleavage within their extracellular domains by metalloproteases to yeild soluble, biologically active protein (Orlinick and Chao, 1998).

Two transmembrane proteins serve as receptors for TNF-α, termed p55 (TNFR I) or p75 (TNFR II) (Schall *et al.*, 1990; Loetscher *et al.*, 1990; Smith *et al.*, 1990 and Nophar *et al.*, 1990). TNFR I has four cysteine-rich domains (CRDs) which are required for efficient ligand binding to TNF-α (Marsters *et al.*, 1992), whereas the Fas receptor requires all three of its CRDs for binding to the FasL (Orlinick *et al.*, 1997). TNF-α binds to both TNFR I and TNFR II and to soluble TNF-binding proteins (which are proteolytic fragments of the full-length receptors shed from the cell surface by proteolytic processing) (Seckinger *et al.*, 1988; Engelmann *et al.*, 1990 and Gantanga *et al.*, 1990). TNFR I has shown a clear role in TNF-mediated cytotoxicity and can stimulate activation of NF-κB (Orlinick and Chao, 1998), whereas TNFR II is thought to act by "ligand passing" to provide increased local concentrations of ligand for TNFR I, which initiates intracellular signalling (Tartaglia *et al.*, 1993).

TNFR-I and Fas (CD95) possess and additional homology in their intracellular domains in a region of about 80 amino acids termed the "death domain". This domain is required for receptor-mediated cytotoxic activity (S. Nagata, 1997 and Itoh and Nagata, 1993) and NF-κB activation (Tartaglia *et al.*, 1993), which mediates many of the pro-inflammatory and immunoregulatory effects of TNFR-I (Baeuerle and Baltimore, 1996). The death domain appears to be a novel protein-protein interaction motif used for the specific recruitment of cellular signalling molecules. TNF and Fas receptors do not possess intrinsic catalytic activity associated with their intracellular domains and thus rely on cytosolic factors to transduce their signals. Several of the interacting signalling molecules, such as FADD/MORT1, TRADD and RIP, have death domains

that are used for interacting with TNFR-I and Fas (Chinnaiyan *et al.*, 1996; Boldin *et al.*, 1995; Hsu *et al.*, 1995 and Stanger *et al.*, 1995). Receptor activation of TNFR-I and Fas occurs by clustering, mediated by the binding of multimeric ligands (Schlessinger and Ullrich, 1992; Engelmann *et al.*, 1990 and Kischkel *et al.*, 1995). After oligomerisation, the Fas receptor recruits the signalling molecule FADD/MORT1 through its death domain (Chinnaiyan *et al.*, 1995 and Boldin *et al.*, 1995). This recruitment, in turn, leads to the recruitment and activation of the caspase proteases FLICE/MACH/caspase-8 and FLICE/caspase-10, which then cleave other caspases to initiate the apoptotic signal-transduction pathway (Boldin *et al.*, 1996; Muzio *et al.*, 1996 and Vincenz and Dixit, 1997). TNFR-I also uses this pathway, recruiting FADD/MORT1 through the adapter protein TRADD (Hsu *et al.*, 1995; Chinnaiyan *et al.*, 1996 and Hsu *et al.*, 1996). TNFR-I can also recruit the protein kinase RIP through the adapter TRADD (Hsu *et al.*, 1996).

Unlike the Fas apoptosis-inducing system, TNF-stimulated cell death often requires the addition of RNA or protein synthesis inhibitors such as actinomycin D or cycloheximide, respectively, to unmask a cell-death activity. This may be a result of the ability of TNFR I and TNFR II to stimulate NF-κB activity, which appears to depend upon signal transduction through the RING-finger protein TRAF2 and the adapter protein TRADD or other cytoplasmic proteins (Hsu *et al.*, 1996c). It has been demonstrated that TNF-α has an enhanced apoptosis-inducing activity when NF-κB activation is blocked (Beg and Baltimore, 1996; van Antwerp *et al.*, 1996 and Wang *et al.*, 1996). NF-κB activation is thought to lead to the production of anti-apoptotic proteins that attenuate the TNFR-I-transmitted death signal.

Relevance to IBD

Pathological effects of TNF are associated with many diseases such as diabetes, childhood chronic inflammatory bowel disease and septic shock. Studies on TNF-α production have detected no significant differences in serum or mucosal specimens between IBD patients and normal controls (Greenfield *et al.* 1993), whereas organ cultures of involved IBD mucosa were found to produce increased amounts of TNF-α compared to normal mucosa (Reimund *et al.* 1996). Tissue levels of TNF-α mRNA were not increased in IBD specimens using PCR amplification (Isaacs *et al.* 1992). It has been suggested that the increase of IL-6 in IBD, which inversely regulates TNF-α production, may be responsible for the apparent decreased expression of TNF-α in intestinal inflammation (Isaacs *et al.* 1992). The systemic levels of TNF-α were not found to be elevated in an experimental model of chronic colitis compared to controls. It has been suggested that other pro-inflammatory mediators with biological properties parallel to those of TNF-α, such as IL-1, may be responsible for the systemic manifestations of chronic colitis (Mack *et al.* 1992). In another study the amount of TNF-α in the stools has been referred as a marker of intestinal inflammation (Braegger *et al.* 1992). TNF-α has a large range of biological activities, most similar with those of IL-1, including the induction of cytokines and enzymes that are involved in the generation of inflammatory mediators (Beutler & Cerami, 1995). In addition, TNF-α has been found to increase adhesion molecule expression on the cell membranes of monocytes and granulocytes and it has been suggested that this cytokine might be responsible for leukocyte recruitment into the bowel wall in IBD (Greenfield *et al.* 1993; Lo *et al.* 1989).

(iii) Interferon (IFN)-γ

Biology

IFN-γ is a noncovalent 34 kDa homodimer (Fountoulakis *et al.*, 1992). It is a multifunctional protein, produced by T cells and natural killer cells, first observed to have

antiviral activity by inhibiting viral replication (Kerr and Stark, 1992). Its functions are largely immunomodulatory and include (i) stimulation of bactericidal activity of phagocytes (ii) stimulation of antigen presentation through class I and class II major histocompatibility complex (MHC) molecules (iii) orchestration of leukocyte-endothelium interactions (iv) effects on cell proliferation and apoptosis, as well as (v) the stimulation and repression of a variety of genes with obscure function (Boehm *et al.* 1997).

Signalling

A published model for the cellular response to IFN-γ (Greenlund *et al.*, 1994) describes the sequential events that follow binding of IFN-γ to its receptor. Initially, binding induces dimerization of the receptor and activation of the Janus tyrosine kinases JAK1 and JAK2. The IFN-γ receptor and an additional protein termed signal transducer and activator of transcription (STAT)1α (or gamma-interferon activating factor (GAF)) are subsequently phosphorylated. Phosphorylated STAT1α dimerizes and possibly in combination with another protein enters the cell nucleus where the complex binds to distinct sites within the promoter of IFN-γ responsive genes, e.g., the gamma-interferon activation site (GAS) and the interferon-stimulated response element (ISRE). This binding induces gene expression and the strength of the response of an IFN-γ activated gene is, in part, dependent on the presence or absence of a positive-acting GAS or negative-acting silencing flanking sequence adjacent to the ISRE. A family of transcription factors, termed interferon regulatory factors (IRFs) recognise ISREs. IRFs are inducible by IFN-γ (Sims *et al.*, 1993) and, interestingly, TNF-α (Fujita *et al.*, 1989). Of note is the induction of iNOS gene depends on IRF-1 (Kamijo *et al.*, 1994).

Many genes that are inducible by IFN-γ are also inducible by TNF-α. This synergy is likely due to the fact that these genes contain an ISRE and an NF-κB-site in their promoter. Also, TNF-α can induce IRF-1 via the NF-κB-site within its promoter (Fujita *et al.*, 1994) and IFN-γ can activate NF-κB (Siebenlist *et al.*, 1994). The anti-inflammatory cytokine, IL-4, exerts antagonistic actions on the induction of many genes induced by IFN-γ (Boehm *et al.*, 1997), e.g., inducible nitric oxide synthase (iNOS) (Liew *et al.*, 1991).

Relevance in IBD

The reports about IFN-γ production in IBD are contradictory (Radford-Smith & Jewell, 1996). Decreased production by intestinal mucosal mononuclear cells in both forms of IBD was initially reported (Lieberman *et al.* 1988). Subsequent reports suggested particular relevance of this cytokine to CD, as indicated by the spontaneous release of IFN-γ and increased IFN-γ mRNA expression by lamina propria mononuclear cells (Fais *et al.* 1991) and the presence of IFN-γ-secreting T cells in actively inflamed mucosa (Breese *et al.* 1993). In other reports, the production of IFN-γ in peripheral blood mononuclear leukocytes of patients with IBD, was found to be the same as that in controls (Nakamura *et al.* 1992). Mitogen activation of peripheral blood mononuclear cells from IBD patients showed normal or decreased levels of IFN-γ (Miura & Hiwatashi, 1985; Stalnikowicz *et al.* 1985; Mutchnick *et al.* 1988). However, in experimental models, inflamed colonic mucosa from mice lacking the G protein, Gαi2, with colitis exhibited increases in IFN-γ and an important role for this lymphokine in IBD has been suggested (Hornquist *et al.* 1997).

Anti-inflammatory cytokines

T-cell-derived cytokines IL-4, IL-10, and IL-13 are thought to have anti-inflammatory effects and changes in their production may be associated with the pathogenesis of IBD (Kucharzik *et al.* 1997).

(i) IL-10

Interleukin-10 is produced by a variety of cells including activated human T-cells. It is a potent suppressor of cytokine and chemokine generation by activated monocytes/macrophages (Moore *et al.*, 1993; Hsu *et al.*, 1990; Vieira *et al.*, 1991; de Waal Malfyt *et al.*, 1991; Fiorentino *et al.*, 1991) and polymorphonuclear cells (PMNs) (Kasama *et al.*, 1994). IL-10 mRNA expression is decreased in the majority of UC patients (Nielsen *et al.*, 1996). IL-10 was able to down-regulate all proinflammatory cytokines in active IBD as well as in controls (Kucharzik *et al.*, 1996 and Murata *et al.*, 1995) and IL-10 deficient mice were found to develop chronic enterocolitis (Kuhn *et al.* 1993; Rennick *et al.* 1997). *In vivo* topical application of IL-10 induces down-regulation of proinflammatory cytokine secretion both systemically and locally (Schreiber *et al.*, 1995).

(ii) IL-4 and IL-13

IL-13 is a potent suppressor of cytokine and chemokine expression by activated monocytes and macrophages (Minty *et al.*, 1993; McKenzie *et al.*, 1993; Zurawski and de Vries, 1994; de Waal Malefyt *et al.*, 1993), and endothelial cells (Marfaing-Koka *et al.*, 1995). In addition IL-13 induces the production of IL-1ra (Muzio *et al.*, 1994) and modulates the expression of cell surface proteins such as class II MHC antigens (de Waal Malefyt *et al.*, 1993). The results concerning IL-13 production in IBD patients are contradictory so far (Radford-Smith and

Jewell, 1996). The inhibitory effect of IL-13 on TNF-α and IL-6 production in differentiated macrophages was diminished in IBD patients and the anti-inflammatory activity of IL-13 was found to be partially reduced in patients with active IBD (Kucharzik *et al.*, 1996). A more detailed discussion of IL-13 can be found in section 1.5.

Similar biological activities are also displayed by IL-4, in addition both cytokines are potent stimulators of B-cell IgE production, while IL-4 alone has an effect on human T-cells (Zurawski and de Vries, 1994). IL-4 mRNA expression is decreased in intestinal tissue from CD patients, while IL-10 mRNA expression is decreased in the majority of UC patients, suggesting different immunopathogenesis of the two diseases (Nielsen *et al.*, 1996). Activated monocytes with increased expression of proinflammatory cytokines play a major role in IBD. It is interesting to note, therefore, that immunoregulatory cytokines such as IL-4 and IL-10 can effectively suppress the proinflammatory response of activated monocytes (Kucharzik *et al.*, 1997). With regard to IL-13 and IL-4, there was no significant suppression of TNF-α and IL-6 in those patients with active IBD (Kucharzik *et al.*, 1996), a finding compatible with an impaired systemic and mucosal anti-inflammatory activity in IBD.

Reactive oxygen and nitrogen metabolites in IBD

Abundant infiltration by PMN leukocytes has long been considered a hallmark of active IBD (Saxon *et al.*, 1990), but the role of these cells in inflammation and tissue injury has not been investigated adequately. The contribution of PMNs to IBD pathogenesis has come under renewed attention after recognition that they are the main source of potent toxic molecules such as reactive oxygen metabolites (ROMs) and reactive nitrogen metabolites (C. Fiocchi, 1998). With the explosion of interest in nitric oxide as a crucial signalling and bioactive molecule in the

gastrointestinal tract, its role in IBD has become the focus of a new investigation. There is evidence from animal models that ROMs are involved in gut inflammation (Keshavarzian *et al.*, 1990) and the same appears true for NO through the modulation of inducible NO synthase (Ribbons *et al.*, 1995). Inhibition of inducible NO synthase by compounds such as L-arginine analogues can significantly decrease the extent and severity of tissue injury in experimental colitis (Rachmilewitz *et al.*, 1995).

These findings from animal models may have parallel in human IBD. Chemiluminescence probes show large quantities of ROMs in the mucosa of patients with CD and with UC that correlate with disease severity (Simmonds *et al.*, 1992). Elevated NO synthase activity was reported initially in UC but not CD colonic tissue, but subsequent studies showed that NO generation and synthase activity are increased in both forms of IBD (Boughton-Smith *et al.*, 1993 and Rachmilewitz *et al.*, 1995). Normal colonic epithelium does not express inducible NO synthase activity, but its expression is induced in the epithelium involved by IBD and other types of inflammation (Singer *et al.*, 1996). The functional role of NO in IBD is far from settled in view of its dual toxic and protective effects (see section 1.4). A complementary aspect to the elevation of ROMs and NO in inflammation is the observation that IBD mucosa is relatively depleted of oxidant defences, rendering it susceptible to oxidative injury (Buffington *et al.*, 1995), and reactive metabolites cause direct epithelial cell damage in active IBD (McKenzie *et al*, 1996). This has led to the assumption that limiting production of these highly reactive molecules may improve IBD. Adding methylprednisolone to mucosal organ culture decreases NO activity (Rachmilewitz *et al.*, 1995), and sulfasalazine, mesalamine and olsalazine have a scavenger effect on superoxide radical formation (Gionchetti *et al.*, 1991). This indirectly suggests that some of the therapeutic effect of drugs commonly used in IBD is mediated by their antiradical activity.

Apoptosis and IBD

Apoptosis is a process of controlled or programmed cell deletion or death and is important in regulating overall cell number in virtually all tissues undergoing cell replication, from invertebrates and developing embryos to adult normal and malignant tissues (A. H.Wyllie, 1987). It is also involved in the removal of damaged cells from the embryo or adult tissue and could be a determinant of carcinogenetic change within a tissue (Kerr *et al.*, 1972). Cells in the human intestinal epithelium (i.e. the mucosal cells lining the gastrointestinal tract) undergo continuous turnover. Rapidly proliferating simple columnar epithelium is restricted to the crypts, which supply cells to replenish the differentiated cells on the surface of the large bowel. In the small and large intestines, new cells originate from stem cells located near the base of the crypts. These differentiate as they migrate away from the crypt base over the course of 3-7 days (Figure 1.3), finally undergoing apoptosis (programmed cell death, PCD) at the mucosal surface (Potten *et al.*, 1997). Apoptosis is a process of fundamental biological importance and will be discussed at greater length in section 1.6. Briefly however, it is suffice to say that there may be a role for dysregulated apoptosis in the pathogenesis of IBD, in that too much cell death could contribute to tissue injury and the disruption of intestinal barrier function resulting in increased permeability (Gardiner *et al.*, 1998). A leaky barrier would intensify antigen absorption, which, in turn, could lead to an exaggerated immune stimulation reflected at the systemic level by an excessive number of mature B cells (C. Fiocchi, 1998). An increased frequency of epithelial apoptosis mediated by the CD95-CD95L system is seen in UC (Sträter *et al.*, 1997 and Iwamoto *et al.*, 1996). Thus, colonic epithelial cell injury, resulting in impaired barrier function, could contribute to the pathogenesis of IBD (Gardiner *et al.*, 1998).

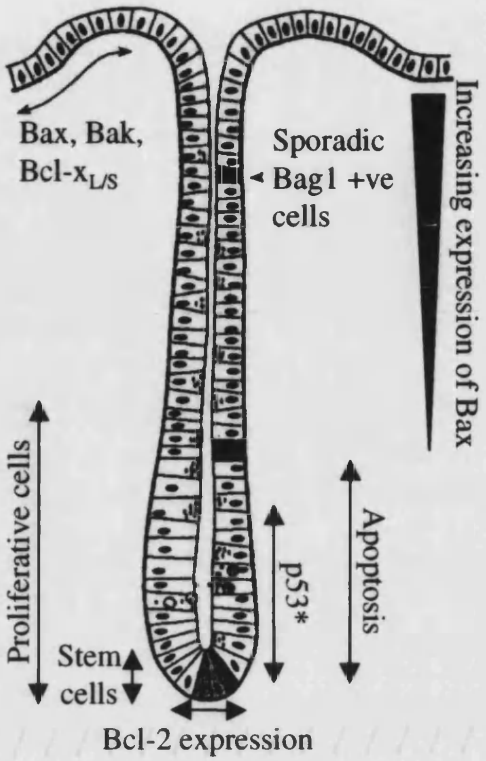


Figure 1.3. Schematic diagram summarising current views on distribution of stem cells, proliferative cells, apoptosis and some gene products affecting apoptosis in the large intestine. Arrows indicate approximate cell positions over which an indicated protein or feature is observed. Wedges indicate gradients of protein expression. * Observations made after irradiation. For more detail on gene products, see section 1.6. Adapted from Potten, 1997.

Summary

The aetiology of IBD appears to be immunologically mediated and factors such as cytokines, reactive oxygen species and nitric oxide all with their inflammatory and regulatory properties appear to play a role in pathogenesis of UC and CD. These molecules have simultaneous protective effects that are important in the resistance to infectious agents and possibly in the healing phase of inflammation. The relative balance of the inflammatory and protective properties of the cytokine network may determine the chronicity of inflammation and the tendency of reactivation of these diseases.

Conclusions

Although the aetiology of IBD remains obscure, it seems most likely that environmental factors, for example dietary or microbial, trigger an inappropriate mucosal inflammatory response in individuals having a genetically abnormal immune system, or dysregulated gut permeability, or irregular mucus and/or colonocyte metabolism. Although the pathogenesis of IBD is gradually being elucidated, we remain ignorant of the mechanisms underlying the chronicity of mucosal inflammation and relapse of quiescent disease in those patients with IBD. For example, is chronicity due to persistence of an exogenous stimulus of inflammation, or to genetically defective down-regulation of the immune response (Podolsky, 1991)? Answers to these questions are a priority if major strides are to be made in the understanding of the pathogenesis of IBD, and in its treatment.

1. 4 NITRIC OXIDE

Background and Chemistry

During the 1980's it was shown that vascular endothelial cells could produce a chemical that resulted in relaxation of the smooth muscle of the walls of the blood vessels. It has been demonstrated that this messenger, previously known as endothelium-derived relaxing factor, is nitric oxide (NO) (Ignarro *et al.*, 1987).

NO is a fairly unreactive inorganic radical, which has diverse biological functions in the cardiovascular, nervous and immune systems. Once formed, NO diffuses to adjacent cells where it activates soluble guanylate cyclase, resulting in the formation of cGMP, which in turn mediates many, but not all, of the biological effects of NO. The specific activation of guanylate cyclase by NO is most likely due to its binding properties to iron hemes. The inherent lack of reactivity of NO makes it a fairly innocuous species unless it is coupled with other radical species, such as superoxide (O_2^-). This chemical property thus allows NO to be utilized as a physiological messenger molecule and, under certain conditions, as a cytotoxic effector molecule as well (J. M. Fukuto, 1995).

Primary reactions of NO (figure 1.4) can result in a variety of secondary products ranging from innocuous nitrate (NO_3^-), nitrite (NO_2^-) and nitroxyl (NO^-) to reactive intermediates such as nitrosonium (NO^+), peroxynitrite ($ONOO^-$) and nitrogen dioxide ($^{\cdot}NO_2$). Reaction of NO with hydrogen peroxide yields singlet oxygen, which is highly toxic (Feldman *et al.*, 1993).

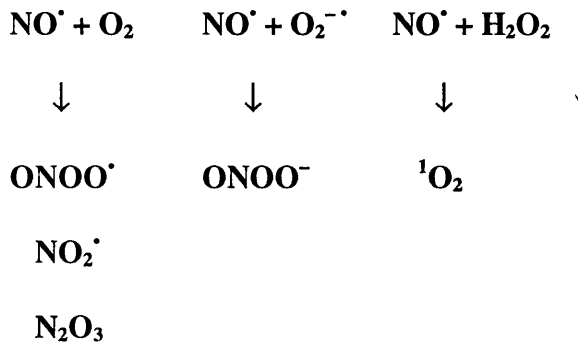


Figure 1.4. Reaction products of NO with oxygen or reactive oxygen intermediates.

NO has been reported to inhibit critical iron-sulfur-containing enzymes involved in mitochondrial respiration , inhibit ribonucleotide reductase , cause ADP-ribosylation of proteins and damage DNA directly. In addition, the production of NO has been implicated in reoxygenation injury following ischaemia , glutamate-mediated neuronal toxicity , inflammation, graft-versus-host disease and as a major arm of host defense against viruses, bacteria and other intracellular parasites (Karupiah *et al.*, 1993).

The number of reports suggesting that NO may promote inflammation-induced cell and tissue dysfunction equals the number of reports demonstrating that NO possesses potent anti-inflammatory properties. The reasons for these apparent paradoxical observations are not entirely clear, however the physiological chemistry of NO and its metabolites may provide clues to help distinguish between the regulatory/anti-inflammatory properties of NO and its deleterious/proinflammatory effects (Grisham *et al.*, 1999 and figure 1.5).

Physiological aspects NO chemistry may be categorised into direct and indirect effects (figure 1.6). Direct effects are those reactions in which NO interacts directly with a

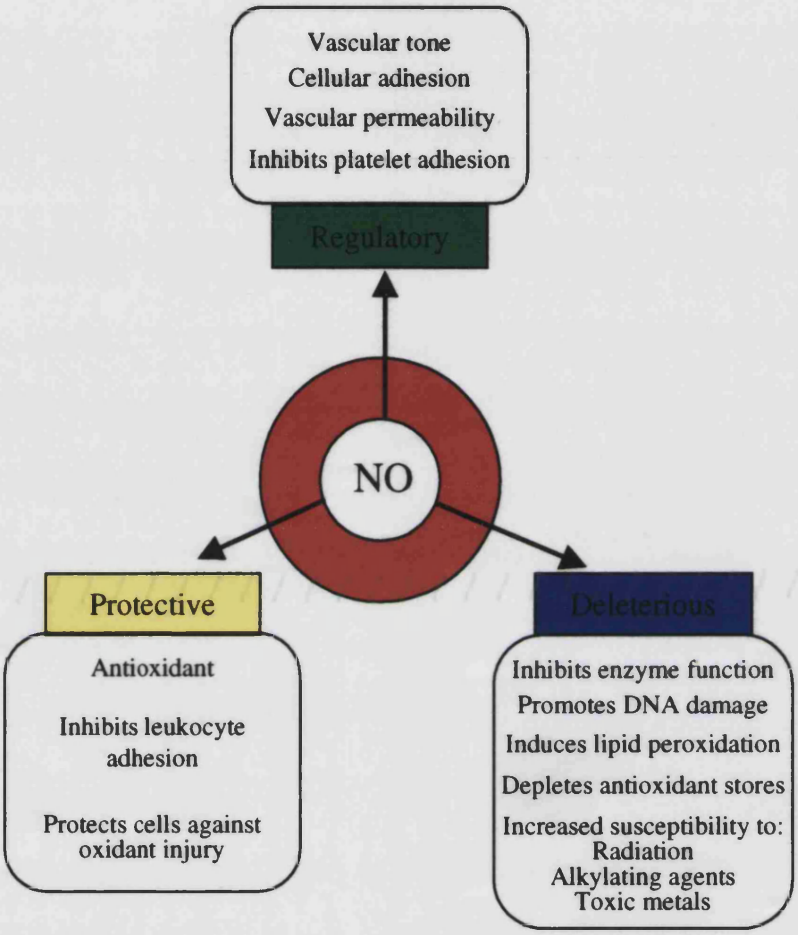


Figure 1.5. Regulatory, protective and deleterious biological effects of nitric oxide.

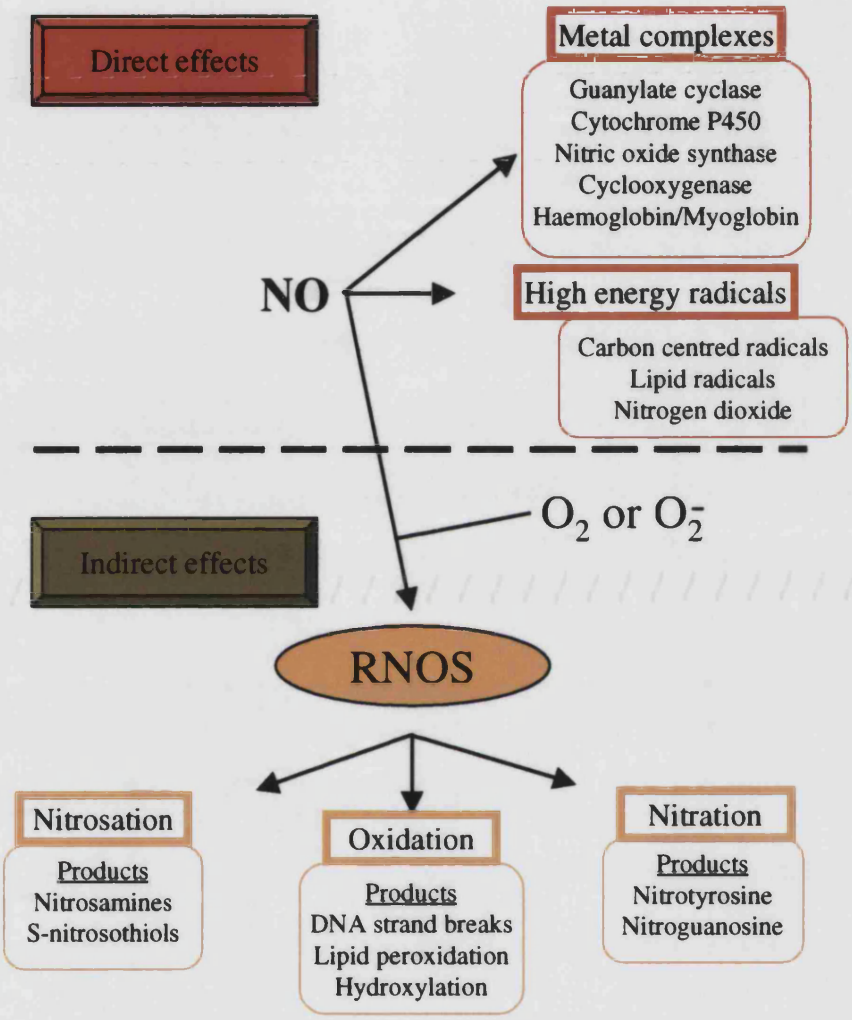


Figure 1.6. Physiological chemistry of NO. RNOS, reactive nitric oxygen species.

biological molecule or target and are thought to occur under normal physiological conditions when the rates of NO production are low. Generally, these types of reactions may serve regulatory and/or anti-inflammatory functions (Grisham *et al.*, 1999). Indirect effects are those reactions mediated by NO-derived intermediates such as reactive nitrogen oxide species derived from the reaction of NO with oxygen or superoxide and are produced when fluxes of NO are enhanced. These types of reactions may predominate during active inflammation. Thus, the relative rates of production, sites of production and steady-state concentrations of reactive species, antioxidants and tissue mediators critically influence the observed apparent toxic or protective effects of NO in biological systems (Crow and Beckham, 1995). The cellular and anatomic sites of production of O_2^- and NO as well as the dominant operative mechanisms of oxidant damage in tissues at the time of O_2^- and NO production, also profoundly influence expression of the differential oxidant injury-enhancing and protective effects of NO. Under pathological conditions NO can reach concentrations of 4×10^{-6} *M*, resulting in competition between O_2^- and NO for the actions of the enzyme superoxide dismutase (SOD) and, therefore, a fraction of O_2^- reacts with NO to produce peroxynitrite. $ONOO^-$ is a potent oxidant as well as a nitrating and hydroxylating agent. Tyrosine residues in proteins are readily nitrated by $ONOO^-$ due to the ability of SOD to selectively enhance nitration in complex media such as the milieu of a cell and nitrated proteins have been implicated in a number of diseases and conditions. This nitration pathway would inhibit tyrosine phosphorylation (Grisham *et al.*, 1999) and may, in some circumstances, mimic phosphorylation in an irreversible manner. By introducing a negative charge onto tyrosine, nitration may alter protein conformation and function, perhaps 'tagging' a protein for proteolysis. Also, nitrotyrosine structurally resembles dinitrophenol, a strongly antigenic compound used for making haptens and endogenous nitration may, therefore, initiate autoimmune processes. In addition, it has been shown that $ONOO^-$ reacts

quite rapidly with zinc-thiolate centres such as those present in numerous transcription factors. Oxidation of such "zinc fingers" could dramatically alter their ability to recognise and bind DNA. Thus, $ONOO^-$ could exert significant effects on gene regulation. (Crow and Beckham, 1995).

Nitric Oxide Synthases

The nitric oxide synthases (NOSs) constitute a family with at least three distinct isoforms. These include the neuronal (nNOS, *NOS1*), inducible (iNOS, *NOS2*) and endothelial constitutive (ecNOS, *NOS3*) NOSs (Nathan and Qiao-Wen, 1994). The three isoforms are distributed across a wide spectrum of cell types and tissues. Furthermore, findings indicate that a cell may express more than one isoform of NOS (Salter *et al.*, 1991), complicating the interpretation of NO derived from any given cell. The NOSs are *P*-450-like hemeproteins (Wang *et al.*, 1993) with a reductase domain at the COOH terminus and an oxidative domain at the NH_2 terminus (figure 1.7). The primary amino acid sequences of NOS isoforms share common consensus sequence binding sites for calmodulin, NADPH, flavin-adenine dinucleotide (FAD) and flavin mononucleotide (FMN) (Wang and Marsden, 1995). Each enzyme functions as a dimeric protein in catalyzing the NADPH-dependent five-electron oxidation of L-arginine to generate NO. L-citrulline is a by-product. Electrons are supplied by NADPH, transferred along the flavins and calmodulin and presented to the catalytic heme centre. The NOS apoenzyme requires tetrahydrobiopterin, prosthetic heme, calmodulin, FMN and FAD as cofactors for monomer assembly and/or catalytic activity (figure 1.8). The nNOS and ecNOS isoforms are constitutively expressed, but enzyme activation requires stimulation of the calcium/calmodulin signaling pathway. The synthesis and release of NO by constitutive NOS isoforms are rapid and do not depend on new protein synthesis. Calmodulin, in the presence of an elevated level of intracellular free

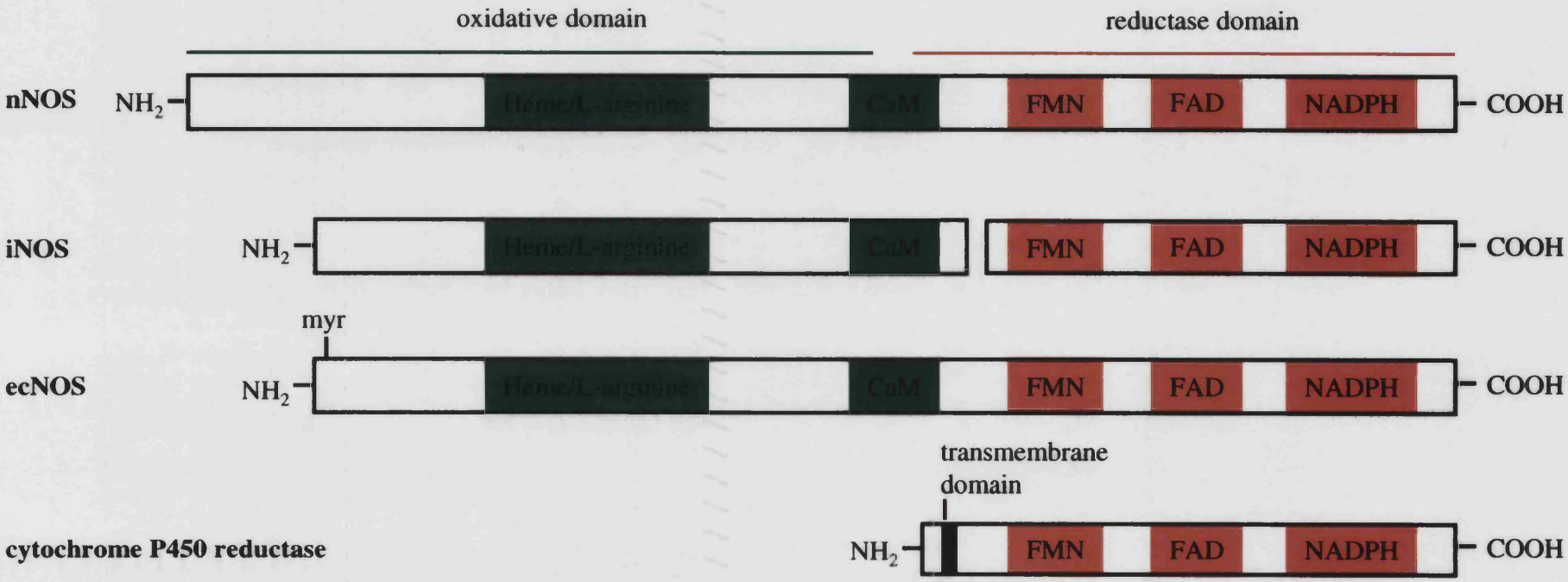


Figure 1.7. Schematic alignment of the deduced amino acid sequences of nitric oxide symthases (NOSs) and the cytochrome P450 reductase. Depicted are consensus binding sites for heme, L-arginine, calmodulin (CaM), flavin mononucleotide (FMN), flavin-adenine dinucleotide (FAD) and NADPH. An NH_2-terminal myristoylation site (myr) is present only on the endothelial constitutive NOS (ecNOS). Neuronal, n; i, inducible.

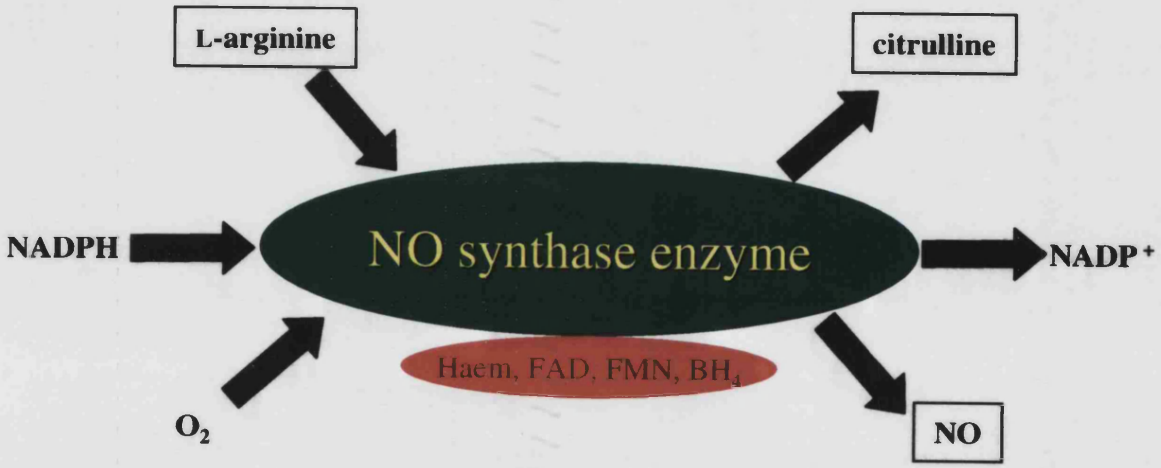


Figure 1.8. Nitric oxide (NO) production from the amino acid L-arginine by the family of enzymes named nitric oxide synthases (NOS).

calcium, activates constitutive NOS activity. In contrast, cytokines or lipopolysaccharide (LPS) stimulates the expression of iNOS over a period of many hours. This process is dependent on new mRNA and protein synthesis Szabo *et al.*, 1995). Once it has been induced, this enzyme produces large amounts of NO and its activity is independent on intracellular calcium levels due, in part, to calmodulin being tightly bound to this enzyme. The iNOS gene is predominantly regulated at the level of transcription by synergistic combinations of proinflammatory cytokines and bacterial cell wall products (Morris and Billiar, 1994). Sequence analysis of the 5'-flanking region revealed consensus sequences that are implicated in cytokine-modulated gene expression, namely, NF-κB, NF-interleukin-6 (IL-6), γ-IRE, a palindromic TNF-RE-like site and a liver-specific transcription factor consensus sequence, AABS. However, there appears to be much variation in the specific requirements of induction, not only between cell types, but also between cells of the same type in different mammalian species. For example, murine macrophages express high levels of iNOS upon exposure to bacterial LPS, with strong synergy occurring when IFN-γ is added (Lorsbach *et al.*, 1993), whereas IL-1 is a potent inducer in chondrocytes, smooth muscle cells, hepatocytes and islet cells (Geller *et al.*, 1993).

NOS inhibitors

A number of strategies have emerged with regard to a pharmacological control of pathological NO production and new therapeutic approaches are studied, that may provide new means for clinical medicine (Pfeilschifter *et al.*, 1996). Since the various isoforms of NOS are distributed in cells and tissues according to their function, there is the possibility that manipulation of NO levels can be accomplished by designing specific pharmacological agents targeted at a single NOS isoform (Fukuto and Chaudhuri, 1995). Since the development of NO as a field of research, multiple NOS inhibitors have been utilised to

characterise the functions of NO as well as attempt to therapeutically intervene in disease processes.

Based on early studies showing that N^{ω}-monomethyl-L-arginine (L-NMMA) blocked macrophage mediated L-arginine-dependent tumour cell cytostasis (Hibbs *et al.*, 1987), a variety of N^{ω}-substituted-L-arginine derivatives have been investigated as potential inhibitors of NOS. ecNOS and iNOS. These show some differences in their affinity for N^{ω}-monosubstituted arginine analogues, which compete with L-arginine for binding sites in the NO synthase enzymes and inhibit their activity Gross *et al.*, 1990 and 1991). Thus, N^{ω}-nitro-L-arginine (L-NNA) and N^{ω}-amino-L-arginine (L-NAA) are more potent inhibitors of ecNOS and iNOS, respectively, while N^{ω}-monomethyl-L-arginine (L-NMMA) was found to be an effective inhibitor of both NOS types. The L-arginine analogues are selective inhibitors of NOS activity, in comparison to other known inhibitors (Knowles and Moncada, 1994).

N^{G}-monomethyl-L-arginine (NMMA) is an amino acid NOS inhibitor, which is recognised by and associates with the substrate binding site for L-arginine (Griffith and Kilbourn, 1996). It is a reversible, competitive inhibitor, which, at 0.1-1.0 mM, is able to inhibit all NOS isoforms substantially, but not completely (>50 %; usually >90 %, depending on levels of L-arginine in the medium).

In 1992 aminoguanidine was described as an inhibitor with high selectivity for iNOS (Corbett *et al*, 1992). Subsequently, aminoguanidine was shown to be as potent as L-NMMA in inhibiting the cytokine-mediated expression of iNOS in cultured insulinoma cells (Hasan *et al*, 1993). In contrast to L-NMMA, which has been shown to be a competitive

inhibitor, it has not been established whether aminoguanidine inhibits iNOS in a competitive manner. Aminoguanidine (AG) is a nucleophilic hydrazine compound that contains chemically equivalent guanidino nitrogens. It is believed that the hydrazine moiety is essential for its selectivity for iNOS without affecting ecNOS (Corbett and McDaniel, 1996). The hemisulfate form is freely soluble in aqueous solution and was the chemical form used in these experiments. Both AG and NMMA display nearly identical inhibitory effects on iNOS activity and are readily taken up by whole cells (Corbett and McDaniel, 1996).

Garvey *et al*, 1997, showed N-(3-(Aminomethyl)benzyl)acetamidine (1400W) to be a slow, tight binding inhibitor of human iNOS. 1400W is a highly selective inhibitor of iNOS *in vitro* and *in vivo* (Knowles *et al*, 1997). Inhibition was either irreversible or extremely slowly reversible and 1000-fold selective for iNOS *versus* ecNOS. The inhibition of ecNOS was rapidly reversible and, thus, relatively inefficient. This inhibitor shows potential as a new therapeutic agent.

NOS and IBD

In the gastrointestinal tract, NO synthesis has been shown to be increased in colonic mucosa from patients with ulcerative colitis (Middleton *et al.*, 1993 and Boughton-Smith *et al.*, 1993) and leukocyte derived NO has been found to induce colonic circular smooth muscle relaxation (Middleton *et al.*, 1991). Active ulcerative colitis is associated with increased vascular permeability and mucosal vasodilatation. In fulminant ulcerative colitis impaired colonic motility is associated with toxic megacolon, which may lead to perforation (Boughton-Smith, 1994). The generation of high levels of NO by the inflammatory stimuli-induced iNOS in the intestinal mucosa and the subsequent formation of reactive products could underlie all these features (Moncada and Higgs, 1993). Indeed, it has been demonstrated that epithelial cells of the colonic mucosa derived from patients with

ulcerative colitis are a rich source of iNOS (Singer *et al.*, 1996 and Kolios *et al.*, 1998). This correlates with the increase in pro-inflammatory cytokines and suggests a specific role of NO in the inflammatory features and symptoms of both ulcerative colitis and Crohn's disease.

1.4 INTERLEUKIN–13

Biology

Interleukin -13 (IL-13) is a pleiotropic cytokine that is secreted by activated Th2 cells with immunoregulatory activities that partially overlap those of IL-4 (Minty *et al.*, 1993 and McKenzie *et al.*, 1993). In B-lymphocytes, IL-13 induces proliferation and differentiation, promotes CD23 expression and production of certain immunoglobulins such as IgG and IgE (Cocks *et al.*, 1993; Defrance *et al.*, 1994 and Punnonen *et al.*, 1993). In monocytes, IL-13 induces morphological changes (McKenzie *et al.*, 1993), up-regulates expression of members of the integrin superfamily and MHC class II antigen expression and down-regulates expression of CD14 and FcγR receptors (Malefyt *et al.*, 1993). In lipopolysaccharide-stimulated monocytes, IL-13 also acts as a suppressor of pro-inflammatory cytokines (eg. TNF-α, IL-1 and IL-6), chemokines (eg. IL-6, macrophage inflammatory protein-1α) and hematopoietic growth factors (eg. granulocyte/macrophage-colony stimulating factor, granulocyte-colony stimulating factor) expression by activated monocytes/macrophages or endothelial cells (Minty *et al.*, 1993 and Malefyt *et al.*, 1993). Another target for IL-13 is epithelial cells and it has recently been demonstrated that IL-13 can modulate IL-8 generation from and inhibit iNOS and COX-2 expression in the human colonic epithelial cell line, HT-29 (Kolios *et al.*, 1996 and 1998 and Jobin *et al.*, 1998). In

addition, IL-13 induces IL-1ra (Yanagawa *et al.*, 1995) and inhibits PKC-triggered respiratory bursts (Sozzani *et al.*, 1995).

Interestingly, IL-13 also exhibits pro-inflammatory functions. For example, IL-13 can activate and inhibit apoptosis in eosinophils (J. E. de Vries, 1998). It induces tissue inflammation, mucous hyperproduction, goblet cell hyperplasia, subepithelial airway fibrosis, crystal deposition, airways obstruction and airways hyperresponsiveness (Zhu *et al.*, 1999). Blockade of IL-13 completely reverses allergen-induced AHR by the reversal of allergen-induced increases in mucous-containing cells in the airways (Wills-Karp *et al.*, 1998).

Receptor structure

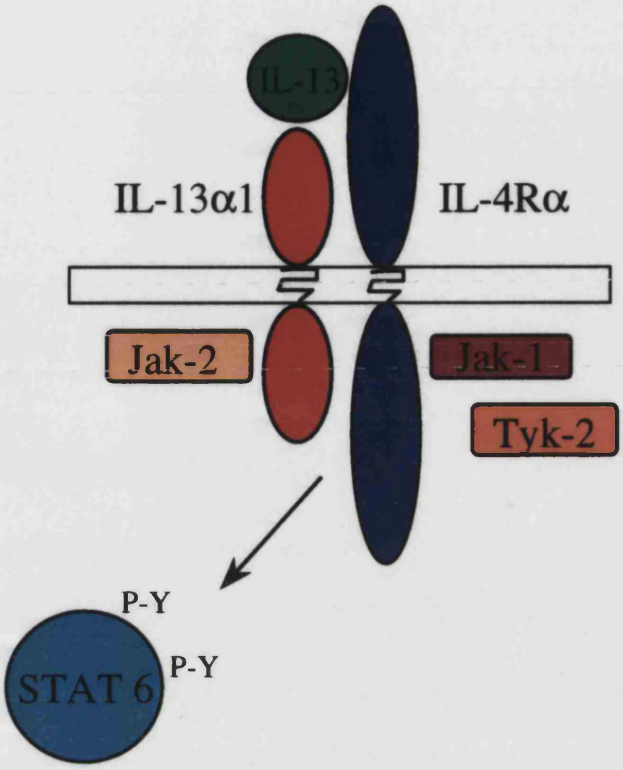
Little is currently known about IL-13 signal transduction, although evidence points to similarities with IL-4 signal transduction. For instance, IL-13 can cross-compete with IL-4 for binding, leading to the suggestion that their receptors (IL-4R and IL-13R) share a common component (Zurawski *et al.*, 1993). Both the IL-4R α chain and the common γ chain subunit have been proposed as likely candidates, (Kondo *et al.*, 1993; Russell *et al.*, 1993; Obiri *et al.*, 1995 and 1997 and Welham *et al*, 1995). A common receptor may explain the observations that IL-4 can mimic every cellular response mediated by IL-13. This is typified in a model of experimental asthma, whereby the asthmatic phenotype is IL-13-dependent and IL-4-independent, but the functional outcome is mediated by the IL-4Rα chain (Grünig *et al.*, 1998). In contrast, human T cells and mouse T and B cells respond to IL-4, but not to IL-13 (Callard *et al.*, 1996).

More recently, two different IL-13R chains have been cloned (Hilton *et al.*, 1996 and Miloux *et al.*, 1997). The IL-13Rα1 is a 65-70 kDa protein and has a 50% homology to IL-5R α chain on a DNA level (Caput *et al.*, 1996). Alone, this receptor binds IL-13 with low affinity, but when complexed with the IL-4Rα chain, shows high affinity binding for both IL-13 and IL-4. IL-13Rα2 binds IL-13 with high affinity (Caput *et al.*, 1996 and Donaldson *et al.*, 1998), although its involvement in signal transduction has not been shown, possibly due to a short cytoplasmic tail. It has been detected in a soluble form, possibly shed from cells (Zhang *et al.*, 1997), but its role in IL-13 biology has not been determined. Thus, there are four potential IL-13R complexes, formed by IL-13Rα21, IL-13Rα2, IL-4Rα and γ chain (Obiri *et al.*, 1997). The type of receptor expressed depends upon the cell and which of the possible receptor components are present. As a result, different cells can display different binding properties for IL-13 and IL-4 (see Table 1.2). A simplified view is depicted in figure 1.9.

Table 1.2 Interleukin-13 Receptor Complexes. For references to the data presented in the table, see text and Obiri *et al.*, 1995 and 1997 and Zurawski *et al.*, 1993 and 1995.

		Ligand-Binding Properties	
IL-13R Complex	**Example**	**IL-4**	**IL-13**
IL-13Rα1, IL-13Rα2	RCC and U251 cells	no competition with IL-13	competes with IL-4
IL-13Rα1, IL-4α	Cos-7, A431 and Colo201 cells	competes with IL-13	high affinity; competes with IL-4
IL-13Rα1, IL-4Rα, γ chain	TF-1 cells	competes with IL-13	competes with IL-4
IL-13Rα1 (low levels), IL-4Rα, γ chain	Raji and MLA-144 cells	binds well to all chains	limited binding; no competition with IL-4

Type I IL-13R
eg. Monkey fbroblasts COS-7,
human epidermoid carcinoma A431,
human ovarian cancer PA-1,
human colon carcinoma cell lines HT-29,
WiDr, COLO201



Type II IL-13R
eg. renal cell carcinoma cell line RCC,
glioblastoma cell line U251,
ovarian cancer cell line IGROV-1.

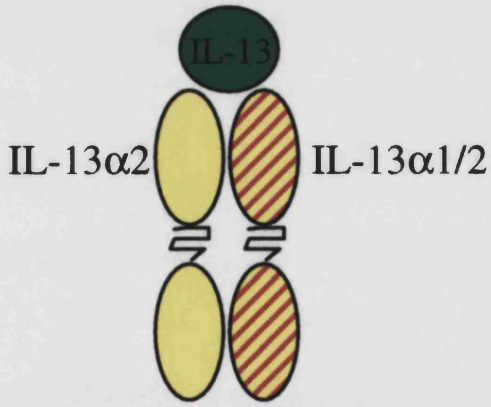


Figure 1.9. IL-13 receptor structure model. In the first model, IL-13R may be composed of heterodimeric proteins of IL-13Rα1 and the140 kDa IL-4Rα chains (4α). Both IL-4 and IL-13 bind to both proteins; however, there is barely detectable IL-13 binding to 4α, while IL-4 binding to α1 is strong and can be detected by cross-linking. Because of the conformation, both IL-4 and IL-13 can cross-compete for each other's binding. In the second model, IL-13 binds to two 65-70 kDa isomers (α1 and α2) and one of these (α1) also binds to IL-4. In this arrangement, IL-13 will have greater binding affinity to α1 than IL-4. Thus, IL-13 can compete for ^{125}I-IL-4 binding; however, IL-4 cannot compete for the ^{125}I-IL-13 binding. It is conceivably possible that there could exist a homodimer of α2, although this has not been shown.

Receptor signalling

The receptors for IL-13 belong to the haemopoietic receptor family, which lack intrinsic tyrosine kinase activity. Unlike other cytokines, IL-4 and IL-13 do not induce tyrosine phosphorylation of the adaptor protein Shc or its association with Grb-2 and they do not activate the mitogen-activated family of protein kinases such as Erk 1 and 2 (Welham *et al.*, 1995). However, Janus family kinases (JAKs) and Signal Transducers and Activators of Transcription (STATs) have been shown to be significant signal transduction components of cytokine receptors (Lin *et al.*, 1995 and J. Ihle, 1996).

Four members of the Jak (Janus kinase or Just another kinase) family have been identified: Jak1 (135 kDa), Jak2 (135 kDa), Jak3 (120 kDa) and Tyk2 (140 kDa). Jak1, Jak2 and Tyk2 are all ubiquitously expressed while Jak3 is found in myeloid cells, natural killer cells and T lymphocytes (J. Ihle, 1996). The Jak proteins consist of 7 Jak homology (JH) domains. JH1 is the kinase domain and contains an important KDYY (Jak3) or KEYY (Jak1, 2 and Tyk2) domain in the catalytic core. Phosphorylation of this site has been associated with an increase in catalytic activity (Hanks *et al.*, 1998; Feng *et al.*, 1997). Indeed, the IL-4Rα chain associates with JAK1, whilst the common γ chain associates with JAK3 and both are required for IL-4 activation of STAT6 (Linn *et al.*, 1995; Ihle *et al.*, 1995; Hou *et al.*, 1994; Palmer-Crocker *et al.*, 1996 and Rolling *et al.*, 1996). Jak1, Tyk2 and STAT6 are also activated by IL-13, but JAK3 is not (Welham *et al.*, 1995).

IL-4 and IL-13 share the ability to induce phosphorylation of several cellular proteins. These include the IL-4α chain itself (Welham *et al.*, 1995 and Smerz-Bertling and Duschl, 1995) and a p170 protein (Welham *et al.*, 1995; Lefort *et al.*, 1995 and Wang *et al.*, 1995), recently identified as insulin receptor substrate-2 (IRS-2) (Sun *et al.*, 1995). IRS-2, like its

homologue IRS-1, contains multiple specific YXXM motifs which, after tyrosine phosphorylation, may bind the SH2 domains of the p85 regulatory subunit of the protein tyrosine kinase/SH2-coupled phophatidylinositol (PI) 3-kinase (Sun *et al.*, 1995 and Songyang *et al.*, 1993). Indeed, several groups have shown that phosphorylation of the p170 kDa protein results in its tight association with phosphatidylinositol (PI) 3-kinase following IL-4 and IL-13 treatment (Welham *et al.*, 1995; Lefort *et al.*, 1995 and Izuhara and Harada, 1993).

The specific role of each receptor chain in IL-13 signalling is unclear. Donaldson *et al.*, 1998 observed that Ba/F3 cells transfected with IL-13Rα1 displayed a mitogenic response to IL-13, but cells transfected with mouse IL-13Rα2 did not. A soluble IL-13Rα2/Fc fusion protein blocked the mitogenic response to IL-13. They suggested that IL-13Rα2 could serve as a dominant negative inhibitor or decoy receptor for IL-13. Wills-Karp *et al.*, 1998 found that administration of IL-13 was sufficient to induce airway hyper-responsiveness (AHR), which was found in allergic asthma. *In vivo* administration of soluble IL-13Rα2 completely reversed IL-13-mediated AHR. Grünig *et al.*, 1998 found that neutralisation of IL-13 activity prevented development of experimental asthma. Both human IL-13Rα1 and IL-13Rα2 genes are located on the X chromosome (Donaldson *et al.*, 1998, possibly suggesting a role in X-linked immune diseases. Research focussing on IL-13 and the IL-13R complex could have significant impacts into the understanding and treatment of X-linked immune diseases, allergen-induced asthma and experimental asthma.

1.5 PHOSPHATIDYLINOSITOL 3-KINASE

Structure and Function

Phosphatidylinositol-3 kinases (PI 3-kinases) are a growing subfamily of lipid kinases that catalyse the addition of a phosphate molecule to the hydroxyl group at the 3-position on the inositol ring of phosphoinositides. Phosphatidylinositol (PI), the precursor of all phosphoinositides, constitutes less than 10% of the total lipid in eukaryotic cell membranes (figure 1.10). However, less than 0.25% of the total inositol-containing lipids are phosphorylated at the 3-position, consistent with the idea that these lipids exert specific regulatory functions inside the cell, as opposed to a structural function (Rameh and Cantley, 1999). To date, nine members of the PI 3-kinase family have been isolated from mammalian cells. They are grouped into three classes, according to the molecules that they preferentially utilise as substrates (figure 1.11), as suggested by Domin and Waterfield, 1997. Four different lipid products can be generated by the different PI 3-kinase members: PI 3-monophosphate (P), PI-(3,4)-bisphosphate (P_2), PI-(3,5)-bisphosphate (P_2) and PI-(3,4,5)-trisphosphate (P_3), respectively (figure 1.10).

PI 3-kinase was first described as a PI kinase activity associated with the viral oncoproteins, v-Src, v-Ros and polyomavirus middle T (Sugimoto *et al.*, 1984). Mutational studies more than 10 years ago indicated a critical role for the associated PI kinase in cell transformation (Courtneidge and Heber, 1987). Subsequent work has confirmed a role for PI 3-kinases and their products not only in growth regulation, but also in various other cellular responses, such as membrane ruffling, chemotaxis and glucose transport {Wennstrom *et al.*, 1994; Wyman and Arcaro, 1994; Turner *et al.*, 1995 and Kimura *et al.*, 1994). More recent

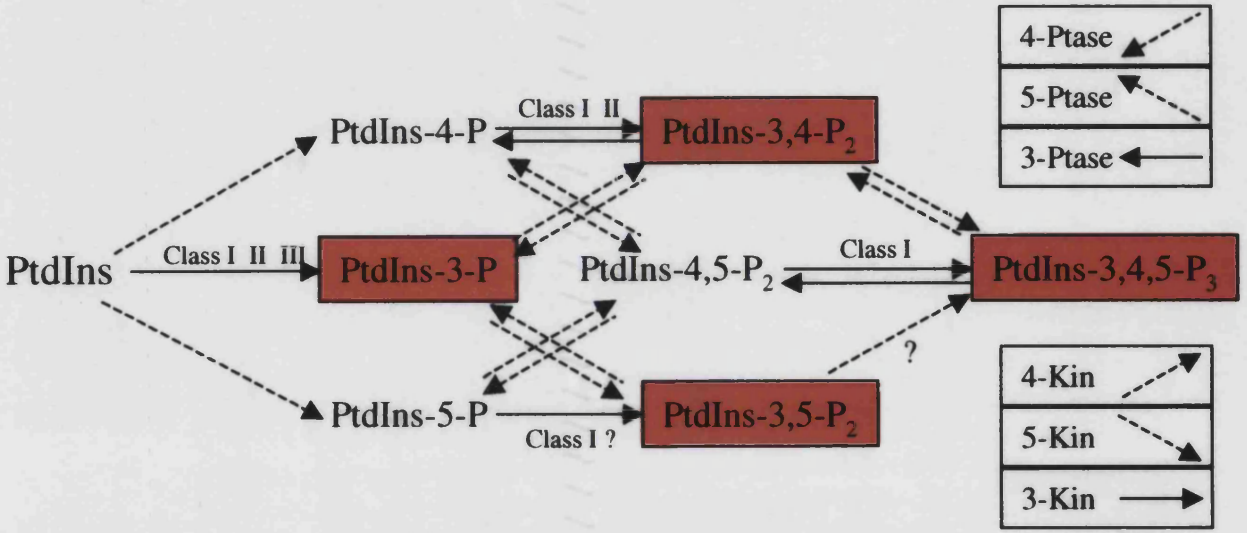


Figure 1.10. The pathways for PI synthesis. The orange boxes indicate the PI 3-kinase products. The classes of PI 3-kinase enzymes that catalyse the phosphorylation of the different PI 3-kinase substrates are indicated on top of the horizontal arrows. Class I enzymes include Class I_A and Class I_B. Kin, kinases; Ptase,phosphatases. Adapted from Rameh and Cantley, 1999.

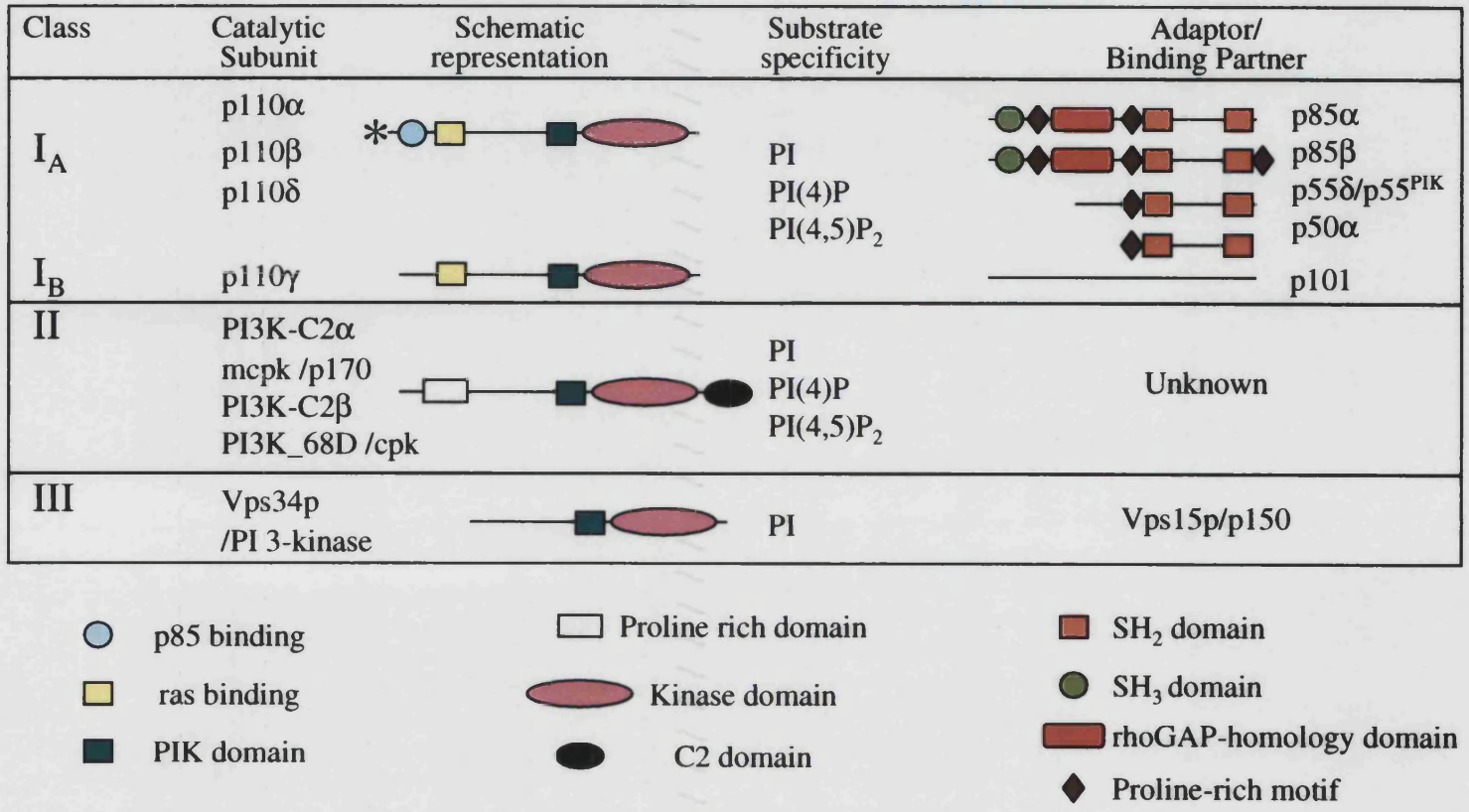


Figure 1.11. The classification of PI 3-kinase family members. The assignment of catalytic subunits to a particular class is based on sequence homology within the catalytic domain (Zvelebil *et al*, 1996). To date, PI 3K-C2α is the only class II member which has been shown to phosphorylate $PI(4,5)P_2$. PI3K_68D is a *Drosophila* protein whilst m-cpk and p170 are murine proteins. The yeast enzyme Vps34p and its human homo;ogue PI 3-kinase can only phosphorylate PI (Volinia *et* al, 1995). The C2 domain was originally defined as the second of four regions within mammalian PKC where it conferred a Ca^{2+} sensitive phospholipid binding (Kaibuchi *et al*, 1980). * p85-interaction region in class IA enzymes. Adapted from Domin and Waterfield, 1997.

findings that PI 3-kinase activation prevents cell death (Franke and Cantley, 1997) have led to increased interest in these enzymes.

There are three classes of PI 3-kinases:

Class I

These PI 3-kinases phosphorylate the phospholipids PI, PI-(4)-P and PI-(4,5)-P_2, although their preferred substrate is thought to be PI-(4,5)-P_2. This class interacts with active GTP-bound Ras and forms heterodimers with adapter proteins (Rodriguez-Vicinia *et al.*, 1994; Kodaki *et al.*, 1994; Rodriguez-Vicinia *et al.*, 1996; Vanhaesebroeck *et al.*, 1997 and Marte *et al.*, 1997). They can be divided into two types (Class I_A and I_B) according to the nature of the interactions with adapter proteins (figure 1.11). The prototypical Class I_A PI 3-kinase consists of an 85 kDa regulatory subunit, which is responsible for the protein-protein interactions via SH2 domain-phosphoprotein interactions, and a catalytic 110 kDa subunit. There are several isoforms of the catalytic subunit and they include the mammalian p110α, β and δ which interact with the regulatory subunits (or p85 proteins, based on the molecular weight of the first two isoforms, α and β). There is no evidence that different regulatory subunit isoforms pair preferentially with different p110 isoforms, however, it may be that different p85 subunits may associate with different subsets of intracellular proteins (Vanhaesebroeck *et al.*, 1997). Class IB PI 3-kinase consists of a distinct p110 catalytic subunit that is stimulated by G-protein $\beta\gamma$ subunits, designated p110γ (Stoyanov *et al.*, 1995). This isoform contains a PIK domain, a kinase domain and a ras-binding domain, but does not interact with p85 proteins. A putative regulatory subunit, p101, has been found bound to p110γ, although regions of interaction have not been mapped (Stephens *et al.*, 1997).

Class II

This class of PI 3-kinases are larger (>200kDa) and phosphorylate only PI and PI 4-P. They contain a synaptotagmin (synaptic vesicle membrane protein) C2 domain in their C-terminal region, which is involved in the Ca^{2+}-dependent binding of proteins to lipid vesicles (A. C. Newton, 1995). However, certain Asp residues important for Ca^{2+} binding are absent in the C2 domains of class II PI 3-kinases and phospholipid binding is Ca^{2+}-independent (MacDougall *et al.*, 1995).

Class III

These PI 3-kinases have a substrate specificity restricted to PI and are homologous to the yeast Vps34p. Vps34p is essential for the trafficking of newly formed proteins from the Golgi to vesicles (Herman *et al.*, 1992 and Shepherd *et al.*, 1996). These PI 3-kinases also appear as heterodimers. Vsp34p forms a complex with a 170 kDa serine/threonine kinase Vsp15p which activates Vsp34p and recruits it to the membrane (Herman *et al.*, 1992). Similarly, human Vps34p associates with a p150 that serves to target PI 3-kinase to a perinuclear position. The current hypothesis is that the class III PI 3-kinases and their PI(3)P lipid product fulfill a housekeeping role in constitutive membrane trafficking and vesicle morphogenesis (De Camilli *et al.*, 1996; Herman *et al.*, 1992 and Shepherd *et al.*, 1996), although class I and II enzymes also have a function in vesicular trafficking, eg., in post-endocytic sorting of ligand-stimulated receptors.

Regulation of D-3 PI lipids

The metabolism of the PI 3-K lipid products is an important point of regulation and evidence indicates that they are not susceptible to hydrolysis by known isoforms of phospholipase (PL) C (Serunian *et al.*, 1989), however, recent evidence suggests there is

regulation at the level of $PI(3,4,5)P_3$ by three distinct cytosolic isozymes of PI-specific PLDs (Ching *et al.*, 1999) that convert $PI(3,4,5)P_3$ to $Ins(3,4,5)P_3$ and phosphatidic acid. More commonly accepted is the notion that different types of lipid phosphatases mediate the major degradative pathway via dephosphorylation. For example, there exist multiple inositide polyphosphate 5-phosphatases that transform $PI(3,4,5)P_3$ to $PI(3,4)P_2$, through which the ratio of these two lipid second messengers is controlled. These enzymes include $PI(3,4,5)P_3$ 5-phosphatases (Jackson *et al.*, 1995 and Woscholski *et al.*, 1995), SHIP (SH_2-containing inositol 5-phosphatase), (Damen *et al.*, 1996; Lioubin *et al.*, 1996 and Drayer *et al.*, 1996) or SIP (signalling inositol polyphosphate 5-phosphatase) (Kavanaugh *et al.*, 1996) and synaptojanin (Woscholski *et al.*, 1997). Especially noteworthy is the recent finding that the tumour suppressor protein, PTEN, is a 3-phosphatase that dephosphorylates $PI(3,4,5)P_3$ (Maehama *et al.*, 1998), thus terminating the second messenger activities of $PI(3,4,5)P_3$ by converting it to $PI(4,5)P_2$.

Class I_A PI-3-kinases

Structure

Three mammalian class I_A PI 3-kinases have been identified to date. p110α and p110β (Hu *et al.*, 1993) appear to be ubiquitously expressed while p110δ (Vanhaesebroeck *et al.*, 1997) is exclusively found in leukocytes. The p110 subunits are 110kDa proteins that interact with the p85 adapter subunits through amino acids 20-108 of their N-terminal domain (Dhand *et al.*, 1994). As mentioned earlier, they contain a ras-binding domain and a C-terminal lipid kinase core. In addition to their lipid kinase activity class I_A PI 3-kinases also possess serine/threonine protein kinase activity. The regulatory p85 subunit is a substrate of this kinase activity and phosphorylation on ser 608 results in a marked decrease in PI 3-kinase's kinase activity (Dhand *et al.*, 1994; Carpenter *et al.*, 1993). Interestingly,

unlike p85 α/β, p110δ is unable to phosphorylate p85 but undergoes autophosphorylation, which inhibits its lipid kinase activity (Vanhaesebroeck *et al.*, 1997). A schematic representation of the PI 3-kinase subunits is shown in figure 1.11.

There are two mammalian isoforms of the p85 adapter subunit α and β. These 85kDa proteins contain two Src homology (nSH2 and cSH2) regions linked by an inter-SH2 domain (iSH2), a Src homology 3 (SH3) domain and a break point cluster region (BCR). SH2 domains mediate protein-protein interactions by binding phosphotyrosine residues within a pYXXM motif (Pawson *et al.*, 1995). However, coupling PI 3-kinase can be mediated through other motifs in T cells (Ward *et al*, 1996). Amino acid residues 479-513 in the inter-SH2 domain have been shown to be the region of p85 that interacts with the p110 subunit (Dhand *et al.*, 1994a; Dhand *et al.*, 1994b; Klippel *et al.*, 1993). The roles of the SH3 and BCR domains are not well defined. SH3 domains bind proline rich regions containing the motif PXXP and, like SH2 domains, are thought to mediate protein-protein interactions. Interestingly, activation of PI 3-kinase via the SH3 domains binding to one proline-rich region (residues 84 to 99) of p85 has been reported (Pleiman *et al.*, 1994).

Receptor Coupling to PI 3-kinase

(i) Direct coupling

The SH2 domains of the p85 regulatory subunit control receptor coupling to PI 3-K. These domains have a high selectivity for binding phosphorylated YXXM sequences, which are present on activated growth factor receptors, such as the PDGF-R. This is followed by an increase in the catalytic activity of the p110 subunit and also targets the signalling complex to the cellular membrane where the PI 3-K substrates are located, although the precise mechanism by which catalytic activity is increased is not fully understood. In the case of

PDGF, binding of ligand to the receptor results in receptor autophosphorylation on tyrosine residues in the YXXM motif of the interkinase domain. PI 3-K is activated following the interaction of p85 SH2 domains with these phosphotyrosine residues (Escobedo *et al.,* 1991; Kazlauska *et al.,* 1990; Coughlin *et al.,* 1989).

(ii) Adapter-mediated coupling

PI 3-K is activated in response to a number of growth factors including IL-3, IL-4, SCF, GM-CSF and IL-5 (Gold *et al.,* 1995). The receptors for these growth factor do not contain YXXM docking sites for p85 SH2 domains. Instead they use adapter proteins to target p85 to the receptor complex, thus activating PI 3-K. For example, insulin activates PI 3-K by recruiting it to the receptor via the insulin receptor substrate (IRS) proteins. IRS-1 (Sun *et al.,* 1991) and IRS-2 (also known as 4PS) (Sun *et al.,* 1995) associate with and are phosphorylated by the intrinsic insulin receptor in the YXXM motif upon activation. This forms a site for p85 SH2 domain association leading, ultimately, to the activation of p110 catalytic subunit (Backer *et al.,* 1992).

Like insulin and also insulin-like growth factor 1 (IGF-1), IL-4 utilises the IRS proteins to target p85 SH2 domains and activate PI 3-K. IL-4 stimulation of T-lymphocytes results in complex formation of Jak1 with both IL-4R and IRS2 and the phosphorylation of all these proteins (*Yin et a.,* 1994). Studies in the haemopoietic cells line FDCP-2 found PI 3-kinase activity associated with the phosphorylated 170kDa IRS-2 protein after IL-4, insulin and IGF-1 stimulation (Wang *et al.,* 1992). When IRS-1 was transfected into these cells it was rapidly and transiently phosphorylated by all three growth factors indicating that IL-4 can also signal through IRS-1 (Wang *et al.,* 1993). In common with the receptors for insulin and IGF-1, IL-4R contains an I4R motif ($PLX_4NPXYXSXSD$) in the juxtamembrane

region, which has been implicated in IRS binding to these receptors. Mutation of Y497 to phenylalanine blocked IL-4-induced IRS-1 phosphorylation and proliferation in IRS-1 transfected 32D cells (Keegan *et al.*, 1994). Taken together, these data point to a mechanism for IL-4 activation of PI 3-kinase, whereby IL-4 binding to the IL-4R results in activation of Jak1 and its subsequent phosphorylation as well as receptor phosphorylation on a number of tyrosine residues including Y497 in the I4R domain. This creates a receptor binding site for IRS-1 and results in phosphorylation in the YXXM motif which is recognised by the p85 adapter subunit SH2 domains, thus activating the p110 catalytic PI3K subunit.

Targets of D-3 PI Lipids

It is generally hypothesised that PI 3-kinase lipid products interact with certain proteins and modulate their localisation and/or activity. The recent characterisation of protein modules, such as pleckstrin homology (PH) domains (Haslam *et al.*, 1993), which can bind lipids, supports this view. Many of the reported downstream effectors of PI 3-kinase contain PH domains. These include PKB (Klippel *et al.*, 1997), PLCγ1 (Falasca *et al.*, 1998), tyrosine kinases such as ITK (August *et al.*, 1997) and Bruton's tyrosine kinase (Salim *et al.*, 1996) and the guanine nucleotide exchange factor Vav (Han *et al.*, 1998). Of the number of putative downstream targets of PI 3-kinase, a key few will be discussed below.

(i) Protein Kinase B

Protein kinase B (PKB, also known as Akt and Rac) was identified in 1991 by three independent groups. Two of these (Jones *et al.*, 1991; Coffer and Woodgett, 1991) identified PKB from it's homology to PKA and PKC hence the names were coined PKB and RAC (related to A and C). The third group (Bellacosa *et al.*, 1991) isolated the cellular

homologue of the vAkt, an oncogene from AKT8, an acute transforming retrovirus isolated from a rodent T-cell lymphoma. PKB is a serine/threonine kinase that has 68% and 73% homology to PKA and PKC, respectively. Three isoforms have been identified PKBα (Jones *et al.*, 1991; Coffer and Woodgett, 1991; Bellacosa *et al.*, 1991), PKBβ (Cheng *et al.*, 1992) and PKBγ (Konishi *et al.*, 1995a) which have approximately 80% homology. PKBα and β encode 60kDa proteins and PKBγ is 55kDa due to a truncated C-terminus. Both PKBα and β appear to be ubiquitously expressed (Konishi *et al.*, 1995b), while PKBγ is found predominantly in the brain and testis and is absent from liver and kidney (Konishi *et al.*, 1995a).

All three isoforms contain an N-terminal pleckstrin homology (PH) domain (residues 1-166). PH domains bind the charged headgroups of phosphatidylinositols and are thought to be involved in mediating interactions with both phospholipids and other proteins. The PH domain is within a region often referred to as the AH (Akt homology) region which spans residues 1-148. The catalytic serine/threonine kinase domain (residues 148-412) lies between the AH domain and the C-terminal tail region.

Activation of PKB

PKB is activated in response to a number of growth factors. These include PDGF (Franke *et al.*, 1995), insulin (Cross *et al.*, 1995; Kohn *et al.*, 1995) EGF, bFGF (Burgering *et al.*, 1995), IL-3, (Franke *et al.*, 1995; Burgering *et al.*, 1995; Songyang *et al.*, 1997; del Peso *et al.*, 1997; Datta *et al.*, 1997; Scheid and Duronio, 1998). Activation of PKB is inhibited by the PI 3-kinase inhibitors wortmannin and LY294002 (Franke *et al.*, 1995), as well as a dominant negative PI 3-kinase mutant (Δp85), (Burgering and Coffer, 1995). Klippel *et al.*,

1997 indicate that the growth factor induced activation of PKB is mediated through lipid products of PI 3-kinase.

Both phosphorylation and the PH domain appear to be important for growth factor induced activation of PKB. In response to growth factor treatment PKB is phosphorylated on T308 (T309 in PKBβ and T309 in PKBγ) in the kinase loop and S473 (S474 in PKBβ, there is no corresponding site in PKBγ due to it's truncated C-terminal) in the C-terminal tail region (Alessi *et al.,* 1996). Mutation of these residues to alanine inhibit the growth factor induced PKB activation while mimicking the negative charge of the phosphate group by mutation to aspartate results in PKB activation in unstimulated cells.

A kinase which phosphorylates PKB on T308 has been identified and named PDK1 (PI-(3,4,5)-P_3 dependent kinase-1) (Alessi *et al.,* 1997). PDK1 is a 63 kDa PH domain containing protein, which is ubiquitously expressed. It is dependent on the PI 3-kinase lipid products PI (3,4) P_2 and PI (3,4,5) P_3 for activation. A kinase (referred to as PDK2) is thought to phosphorylate S473 *in vivo* is yet to be identified, although MAPKK2 is able to phosphorylate this site *in vitro* but is unlikely to do so *in vivo* (Alessi *et al.,* 1996). Balendran *et al.*, 1999 have presented evidence that PDK1 can be converted to a form that phosphorylates both S473 and T308 via interaction with a small peptide corresponding to the carboxy-terminal region of protein kinase C-related protein kinase-2 (PRK2). These observations suggest that PDK1 and PDK2 may be the same enzyme, the specificity of PDK1 towards T308 and S473 being regulated through its interaction with other cellular components (Balendran *et al.*, 1999).

The PH domain is essential for the activation of PKB in a number of systems (Datta *et al.*, 1996; Franke *et al.*, 1997; Datta *et al.*, 1995; Andjelkovic *et al.*, 1996) but in some systems it does not appear necessary (Kohn *et al.*, 1996). The PH domain binds with high affinity and specificity to both PI (3,4) P_2 and PI (3,4,5) P_3 (Franke *et al.*, 1997), although the interaction with PI (3,4) P_2 is about ten-fold weaker (James *et al.*, 1996). The oncogene *vAkt* is located at the cell membrane due to an N-myristylated gag fusion and is constituatively active (Bellacosa *et al.*, 1991). Targeting PKB to the cellular membrane by the fusion of the src myristylation sequence results in the constitutive activation of PKB and the phosphorylation of both T308 and S473 (Kohn *et al.*, 1996). Therefore, binding of the PH domain to the phospholipids in the cell membrane is thought to mediate the translocation of PKB to the membrane and alter the conformation of PKB, allowing access of its kinases to phosphorylation sites (J. Downward, 1998 and Alessi and Cohen, 1998). PDK1 also binds both phospholipid products of PI 3-kinase through its PH domain (Anderson *et al.*, 1998), thus targeting this kinase to the membrane into proximity of PKB, facilitating its activation (figure 1.12).

Downstream Effects of PKB

PKB phosphorylates serine and threonine residues which lie within the consensus sequence RXRZYS/THyd, where Y and Z are small residues (not G), X is any amino acid and Hyd is a bulky hydophobic residue (Alessi *et al.*, 1996). Direct downstream substrates of PKB that have been identified to date are listed in Table 1.3 below. The glycogen synthesis upregulator glycogen synthase kinase-3 (GSK-3) (Cross *et al.*, 1995) and the proapoptotic Bcl-2 family member Bad (del Peso *et al.*, 1997; Datta *et al.*, 1997; Scheid and Duronio, 1998) were the first targets to generate interest in the role of PKB in cellular function and survival. GSK-3 is inactivated by the PKB mediated phosphorylation of Ser9 (Cross *et al.*,

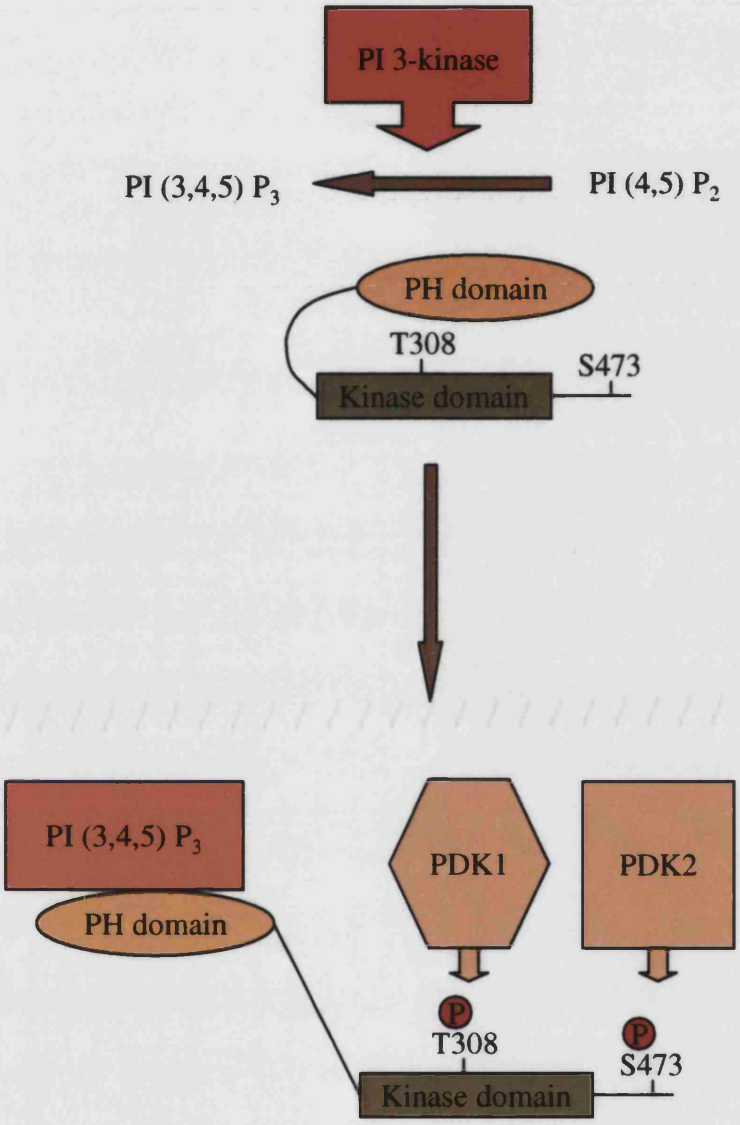


Figure 1.12. The mechanism of activation of PKB. Stimulation of PI 3-kinase leads to an increase of PI (3,4,5) P_3 at the plasma membrane. PKB then interacts with PI (3,4,5) P_3 and/or PI (4,5) P_2, the immediate breakdown product of PI (3,4,5) P_3, through its PH domain, and is thus recruited from the cytosol to the plasma membrane. The interaction of PKB with PI (3,4,5) P_3 alters its conformation so that Thr308 and (perhaps) Ser473 becomes accessible for phosphorylation by PDK1 and PDK2, respectively. Phosphorylation at Thr308 and Ser473 activates PKB. The existence of PDK2 is hypothetical. P, phosphorylation. Adapted from Alessi and Cohen, 1998.

1995; Shaw *et al.*, 1997). Inactivation of GSK-3 results in the activation of glycogen synthase and the subsequent increase in glycogen synthesis.

PKB has been implicated in providing cells with a survival signal. Studies using PI 3-kinase inhibitors and mutants have shown this signal to be mediated through PI 3-kinase (Dudek *et al.*, 1997; Kauffmann-Zeh *et* al., 1997; Kennedy *et al.*, 1997; Songyang *et al.*, 1997; Khwaja *et al.*, 1997). The second direct substrate of PKB, Bad, is a member of the Bcl-2 family of proteins, which are intracellular regulators of programmed cell death (apoptosis). When phosphorylated on a critical serine residue (^{136}Ser) by PKB, Bad is then bound preferentially to a cytosolic protein termed 14-3-3 (Zha *et al.*, 1996). This sequestration of Bad by phosphorylation results in the loss of its pro-apoptotic activity (Datta *et al.*, 1997). The protease, caspase 9, is phosphorylated by PKB, which suppresses its pro-apoptotic function (Cardone *et al.*, 1998).

If PKB is a central mediator of cell survival, it may phosphorylate and regulate the activity of transcription factors that control death genes, such as CD95L (Fas ligand). Indeed, it has been found that PKB-induced phosphorylation and subsequent inhibition of the formation of the CREB/CBP complex modulate the activity of the nuclear factor, cAMP response element-binding (CREB) (Du and Montminy, 1998). In addition, the phosphorylation of a subunit of human telomerase reverse transcriptase (hTERT) enhances the activity of human telomerase (Kang *et al.*, 1999).

PKB regulates the activity of members of the Forkhead family of transcription factors (Brunet *et al.*, 1999 and Kops *et al.*, 1999). PKB phosphorylates FKHRL1, leading to its association with 14-3-3 proteins and its retention in the cytoplasm. Dephosphorylation by

the withdrawal of survival factors results in the nuclear translocation of FKHRL1 where it targets gene activation (Nakae *et al.*, 1999; Tang *et al.*, 1999; Rena *et al.*, 1999 and Guo *et al.*, 1999).

Table 1.3. Substrates phosphorylated by PKB. See text for details.

PROTEIN TARGET	FUNCTION	REFERENCE
H2B histone	Nucleosome structure	Alessi et al., 1996
Glycogen synthase kinase 3α/β	Glycogen synthesis	Franke et al., 1997
6-phosphofructo-2-kinase	Glycogen synthesis	Toker and Cantley, 1997
BAD	Cell survival	del Paso et al., 1997
Caspase-9	Cell survival	Cardone et al., 1998
CREB	Cell survival	Du and Montminy, 1998
FKHRL1/AFX	Cell survival	Brunet et al., 1999
hTERT	Cell survival	Kang et al., 1999
eNOS	NO production	Fulton et al., 1999
	Cardiovascular homeostasis	Dimmeler et al., 1999

(ii) p70 S6 kinase

This kinase becomes activated upon mitogenic stimuli and plays an important role in the progression of cells from the G1 to S phase of the cell cycle. It phosphorylates the S6 protein component of the 40S ribosomal subunit during mitogenic responses, but might also be involved in the regulation of other cellular process (R. L. Erikson, 1991). The role of S6 phosphorylation seems to correlate with an increase in translation from specific mRNAs encoding proteins essential for G1 progression (Kozma and Thomas, 1994).

Activation of p70 S6 kinase is regulated by multiple independent serine/threonine-directed phosphorylations. This activation is independent of the Raf/MAPK pathway, but involves PI 3-kinases and the PIK-related kinase, mTOR (for mammalian target of rapamycin), as well as PKC and proline-directed kinases such as mitogen-activated protein kinases (MAPK). PDK1 can also phosphorylate the activation loop of p70 S6 kinase (Alessi *et al.*, 1997 and Pullen *et al.*, 1998), which is essential for activation.

(iii) PKC isoforms

Both the lipid substrates and products of PI 3-kinases have been reported to activate, *in vitro*, a broad panel of PKC family members, namely the novel isoforms δ, ε and η and the atypical isoform ζ (Chou *et al.*, 1998 and Le Good *et al.*, 1998). It is likely that the activity of these isoforms is affected by PDK1 phosphorylation (ε and ζ) as well as by a direct interaction with PI (3,4) P_2 and PI (3,4,5) P_3 (ε). Inhibition of PI 3-kinase blocks insulin-dependent activation of PKCζ *in vivo* (Standaert *et al.*, 1997 and Mendez *et al.*, 1997) and PDGF-dependent membrane recruitment and activation of PKCε *in vivo* (Moriya *et al.*, 1996). However, the enzymatic activity of PDK1 is not dependent on phosphoinositides (Alessi *et al.*, 1998) and the requirement of PI 3-kinase for PDK1-dependent phosphorylation probably reflects a role for PI (3,4) P_2 and PI (3,4,5) P_3 in co-localising PDK1 and its substrates at specific membranes (Alessi *et al.*, 1998).

(iv) Others

The PH domain of the Bruton's tyrosine kinase (Btk) was shown to interact with PI (3,4,5) P_3 and its head group, inositol 1,3,4,5-P_4, with high affinity (Fukuda *et al.*, 1996; Salim *et al.*, 1996 and Rameh *et al.*, 1997). Phosphorylation of Btk by Src family kinases leads to its activation (Li *et al.*, 1997) and PI (3,4,5) P_3 is involved with modulating this and the

subsequent elevation in cytosolic calcium in response to this B cell stimulation, perhaps by recruiting PLCγ to the membrane (Scharenberg *et al.*, 1998).

Wheres PI 3-kinases phosphorylate PI (4,5) P_2, PLCγ hydrolyses this lipid to produce diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (IP_3) and hence mediates intracellular calcium release and PKC activation (Rhee *et al.*, 1997). Recent work has revealed the existence of cross-talk between PI 3-kinase and PLCγ signalling. The activity of PLCγ is enhanced by 3-phosphoinositides both indirectly (via Btk-related tyrosine kinases, see above) and directly through binding of PI (3,4,5) P_3 to the amino-terminal PH domain and the tandem SH2 domains of PLCγ (Bae *et al.*, 1998; Rameh *et al.*, 1998 and Falasca *et al.*, 1998).

The protein Grp1 (general receptor for inositides) has a PH domain very selective for PI (3,4,5) P_3, which regulates Grp1 by recruiting it to membranes where Arf is localised (Klarlund *et al.*, 1998). These small G proteins are involved in vesicle budding, thus PI (3,4,5) P_3 may regulate coating and budding of intracellular vesicles (Klarlund *et al.*, 1997 and 1998.

PI 3-kinase has a role in chemotaxis and membrane ruffling by activating GTP exchange factors such as Vav2 (Han *et al.*, 1998). The consequent binding of Rac to GTP (Hawkins *et al.*, 1995) may explain the mechanism by which PI 3-kinase is involved in growth factor- and Ras-stimulated cytoskeletal rearrangements that lead to cell migration (Rameh and Cantley, 1999).

The transcription factor E2F, cyclin D3, $p27^{kip1}$ and Rb are all components of the cell cycle machinery and Brennan *et al.*, 1997 established a crucial link between PI 3-kinase/PKB activation and progression into the cell cycle by the modulation of the activity of these critical components. E2F is known to have an important role in the regulation of apoptosis and cell cycle progression (Field *et al.*, 1996 and Yamasaki *et al.*, 1996).

1.6 APOPTOSIS

Background

The concept that accumulation of unwanted cells is due to uncontrolled cell proliferation has been amended. Cells also accumulate when proliferation rates are normal but death rates are lower. The realisation that cell death is a normal process in development and cellular homeostasis has opened a completely new avenue for exploration of the causes and treatments of disease (Kerr and Wyllie, 1972).

Failure of cells to die is an integral mechanism in some cancers and autoimmune disorders. Conversely, an abnormal increase in cell death is observed in neurodegenerative disorders and ischaemic injury. During development the number of cells differentiating into a cell type designed to perform specific functions often exceeds the number of cells required to perform the functions (e.g., innervation) (R. W. Oppenheim, 1991). The superfluous cells are eliminated through cell death (Nagata and Goldstein, 1995 and Jacobson *et al.*, 1997). A miscue in this process is detrimental to the development of the organism.

Pathogens have evolved molecules that affect the death pathway. HIV appears to induce cell death by attacking the molecular machinery involved in inhibiting cell death (Strack *et*

al., 1996). Other pathogens preserve their host by inhibiting the induction of a cell death pathway. In eukaryotic multicellular organisms a mechanism has evolved that eliminates unwanted cells with minimal disturbance to the organism. This process of "programmed cell death" or "cell suicide" is called apoptosis (figure 1.13). The apoptotic elimination of unwanted cells is through a program engrained within the targeted cell (Weil *et al.*, 1996). The suicide program leads to partial autodigestion of intracellular components, with the cell splitting into plasma-membrane limited vesicles called apoptotic bodies. The lipid distribution between the outer and inner leaflets of the plasma membrane enclosing the apoptotic bodies is critical for elimination of these cellular remnants. Phosphatidylserine, normally present in the inner leaflet and excluded from the outer leaflet, is exposed in the outer leaflet of apoptotic bodies. The presence of phosphatidylserine in the outer leaflet serves to mark apoptotic bodies for elimination by phagocytic cells (Fadok *et al.*, 1992). Thus, the process of apoptosis does not elicit an inflammatory response. This is in marked contrast to the inflammatory response during necrosis, where loss of plasma membrane integrity and release of cytoplasmic contents into the surroundings accompanies cell death and provokes an inflammatory response. For a comparison of distinguishing features, see table 1.4.

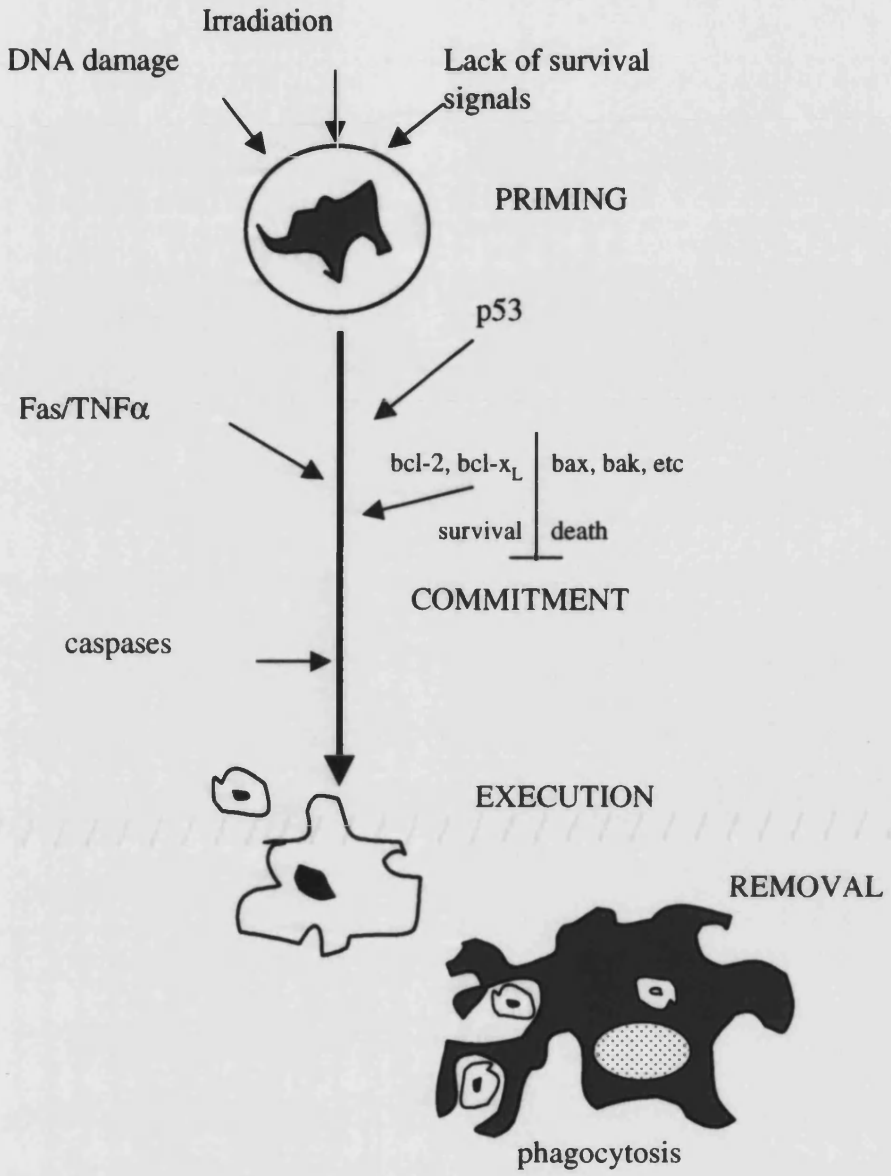


Figure 1.13. Schematic representation of the process of programmed cell death (apoptosis). Cells committed to undergo apoptosis condense and fragment into apoptotic bodies which are removed by neighbouring cells and macrophages. See text for more detail.

Table 1.4. Distinguishing features between apoptosis and necrosis.

	APOPTOSIS	NECROSIS
Causes	Lack of growth factors Hormonal influences Mild toxic influences	Anoxia Physical damage Chemical damage
First apparent cellular changes	Shrinking Convolution	Swelling
Nuclear changes	Condensation, segmentation DNA fragmentation	
Cell membrane changes	Surface protrusions, budding Changes in PS distribution	Smoothing, lysis
Mitochondrial changes	Release of cytochrome *c*	Swelling
Metabolic/Synthetic changes	Active changes in gene expression (eg., Bcl2, Bax); Active protein synthesis; Protease activation	

Induction

Metazoan cells appear to be programmed to die by default and they undergo apoptosis if they do not receive appropriate survival cues from their environment. In addition, metazoan cells have internal sensors for well-being that can initiate apoptosis if the cell is unable to repair defects such as DNA damage. For example, the p53 protein is essential for the induction of apoptosis as a response to chromosomal damage, e.g. due to γ-irradiation. It acts by blocking DNA replication of damaged cells. If the chromosomal lesions cannot be repaired within a certain period of time, the cells die by apoptosis. Cells deficient for p53 replicate in spite of the DNA damage, which favours the accumulation of further mutations and reduces the efficiency of chemotherapeutic drugs (Agarwal *et al.*, 1998).

Higher metazoans have evolved an additional signalling mechanism that actively directs cells to die by apoptosis. For example, certain hormones like glucocorticoids can induce apoptosis (figure 1.14). This pathway appears to be particularly significant in the case of thymocytes and can explain at least in part the immunosuppressive effect of the glucocorticoids. How these drugs induce apoptosis is not exactly known but it is reasonable to assume that it is the well-established gene regulatory activity of the glucocorticoid receptor complex, which is responsible for this effect (King and Cidlowski, 1998).

This type of 'instructive apoptosis' is critical in the immune system where the deletion of activated lymphocytes at the end of an immune response or the elimination of virus-infected cells or cancer cells by cytotoxic lymphocytes is important. Granzyme B, also known as CTL proteinase-1, fragmentin-2, and RNKP-1, is a serine protease which is released by cytotoxic T-cells as part of the cytolytic granules upon a specific interaction with a target cell (figure 1.14). Delivery of granzyme B to the cytoplasm of a target cell is sufficient for the induction of apoptosis, a hallmark of CTL killing. Perforin is a constituent of the cytolytic granules and is capable of forming transmembrane pores in a process that requires calcium. These pores are probably responsible for membrane damage but appear not to be sufficient to kill a cell. In fact, it is assumed that a major function of these pores is to facilitate the entry of granzyme B (J. A. Trapani, 1995).

Cytokine-related mechanisms of apoptosis can be sub-divided into two pathways: (i) positive induction by ligand binding to a plasma membrane receptor and (ii) negative induction by loss of a suppressor signal (see figure 1.14 for a schematic view of the apoptotic pathways).

(i) Positive Induction of Apoptosis

The tumour necrosis factor (TNF) family of receptors (TNFR) is characterised by homology in the extracellular domains. Some of these receptors initiate apoptosis, some initiate cell proliferation and some initiate both. Signalling by this family requires clustering of the receptors by trimeric ligand and subsequent association of proteins with the cytoplasmic region of the receptors (Jones *et al.*, 1989).

The TNFR family contains a sub family with homologous cytoplasmic 80-amino-acid domains. This domain is referred to as a death domain (DD), so named because proteins that contain this domain are involved in apoptosis.8 The distinction between members of the TNFR family is exemplified by two TNFRs coded by distinct genes. TNFRI (55 kDa) signals both the initiation of apoptosis and the activation of the transcription factor NFkB. TNFRII (75 kDa) functions to signal activation of NF-kB but not the initiation of apoptosis. TNFRI contains a DD; TNFRII does not (S. Nagata, 1997).

Other members of the TNFR family with DD are Fas/APO-1/CD95 and DR-3 (Ashkenazi and Dixit, 1998). Aggregation of these receptors initiates apoptosis. Aggregation of the receptors by trimeric ligand orients the DD in a conformation that recruits adapter proteins. The adapter proteins also contain a DD and association of adapters with the receptor is via a homotypic DD interaction. As will become obvious below, homotypic domain interactions between proteins involved in revving up the cell for self-destruction is a common theme of apoptosis.

Aggregation of TNFRI through association with trimeric ligand induces association of the adapter proteins TRADD (TNFR associated protein with a DD) and FADD (Fas associated

protein with a DD) (Hsu *et al.*, 1996 and Chinnaiyan *et al.*, 1995). FADD also contains a 'death effector domain' (DED). The DED of FADD recruits the zymogen form of the cysteine protease FLICE/Machα1. FLICE/Machα1 zymogen contains a large pro-region that is homologous to the DED of FADD (Muzio *et al.*, 1996 and Boldin *et al.*, 1996). This pro-region is removed during activation. The large DED-containing pro-region appears to distinguish FLICE/Machα1 from most of the other cysteine proteases that contain small, DED-lacking pro-regions, suggesting that the proteases activated earliest in the apoptotic proteolytic cascade have large DED-containing pro-regions.

TNF can also induce activation of NF-kB when bound to TNFRI . The adapter TRADD has at least two binding domains, DD and TRAF (TNFR associated factor), of approximately 280 amino acids located near the N-terminus and C-terminus, respectively (Hsu *et al.*, 1996). DD causes TRADD to associate with TNFRI, and TRAF, through homotypic TRAF do main interactions, recruits TRAF-2. The C-terminus of TRAF-2 contains a "ring domain" that binds and activates NFkB (Hsu *et al.*, 1996 and Rothe *et al.*, 1995). NF-kB is then transported into the nucleus where it promotes transcription.

The TNFRII subgroup receptors contain a cytoplasmic domain different from TNFRI. Oligomerisation of TNFRII leads to the binding of two proteins, TRAF-1 and TRAF-2, which form an oligomer through homotypic interaction of their TRAF domains (Rothe *et al.*, 1995). TRAF-2 then binds and activates NFkB. CD40-mediated activation of NFkB proceeds through a similar pathway except that an additional protein, TRAF-3, is involved (Hu *et al.*, 1994).

Fas/APO-1/CD95-initiated cytosolic adapter complex is similar to the complex formed on the cytoplasmic domains of aggregated TNFRI. An additional protein, RIP (receptor interacting protein), has been identified in the complex formed on Fas (Stanger *et al.*, 1995). RIP contains a DD at its N-terminus and a serine kinase-domain at its C-terminus (Hsu *et al.*, 1996). The function of the kinase is unknown. RIP also interacts with and activates NF-kB. There is an adapter protein named RAIDD (RIP-associated ICH1 homologous protein with a DD) that binds to the pro-region of ICH1 and links RIP to the cysteine protease ICH1 (Duan and Dixit, 1997).

Similar to the complex formed on TNFRI, the DED of the adapter FADD binds the DED present in the pro-region of the cysteine protease FLICE/Machα1 (Boldin *et al.*, 1996). Thus, there appears to be redundant pathways by which apoptosis can be initiated by a single receptor. RIP has also been found associated with the TNFRI (Duan and Dixit, 1997). The pro-region released after proteolytic cleavage of FLICE/Machα1 has been found on the DD complex associated with Fas (Boldin *et al.*, 1996). It appears that active FLICE/Machα1 is released to initiate the cysteine protease cascade, with the DED-containing pro-region remaining with the complex.

Many of the proteins in the cytoplasmic apoptotic-inducing complexes have been identified, but their regulation is unknown. Many are phosphorylated, but significance of post-translational modifications is unclear. Other proteins also associate with the cytoplasmic receptor-associated complexes. Two mammalian proteins, c-IAP-1 and c-IAP-2 (cellular inhibitor of apoptosis), are found associated with TRAF-1, TRAF-2 and TNFRII cytoplasmic complex (Duan and Dixit, 1997). These proteins contain regions homologous to the baculoviral inhibitors of apoptosis (all contain BIRs, baculovirus IAP repeats whose

functions are unknown). Clarification of the functions of these proteins and the identification of other complex-associated proteins will undoubtedly aid in dissecting the involvement and functions of proteins involved in direct activation of apoptosis and of other signalling events that depend on the TNFR family.

(ii) Loss of Cytokine-Dependent Suppression of Apoptosis

The viability of many cells is dependent on a constant or intermittent supply of cytokines or growth factors. In the absence of the factor, the cells under go apoptosis. The Bcl-2 family of proteins are central components to apoptosis resulting from the absence of incoming signals generated by cytokine binding. Over-expression of some family members (e.g., Bcl-2 and Bcl-xL) suppresses apoptosis when cytokines are withdrawn. Over-expression of other members (e.g., Bad, Bax and Bik) override the incoming signals from the cytokine-receptor and induce apoptosis. The suppresser members of the Bcl-2 family form homodimers or heterodimers with inducer-type members. Suppressor members prevent apoptosis when homodimerised but are ineffective in protecting from apoptosis when heterodimerised with an inducer member. Thus, the dimerisation state of the suppressor members of the Bcl-2 family is one determinant of cellular life or death. Recent reports have begun to unite the Bcl-2 family of proteins with other components into a single pathway that, although similar in many aspects to the TNF/Fas pathway, is unique and provides an additional method to eliminate unwanted cells (Chao and Korsmeyer, 1998).

Molecular mechanisms

The molecular mechanisms of apoptosis caused by withdrawal of cytokines may be best understood by starting with the roles of the Bcl-2 family members. The suppressers Bcl-2 and Bcl-xL are integral membrane proteins (Nguyen *et al.*, 1993) found as dimers facing the

cytosol. They are predominantly located in the outer mitochondrial membrane, with less in the endoplasmic reticular and nuclear membranes (Krajewski *et al.*, 1993). Dimerised Bcl-2 or Bcl-xL have regions that bind to a protein that, in mammalian cells, is the homologue to CED-4, an adapter protein identified in the nematode *Caenorhabditis elegans* (Wu *et al.*, 1997 and Chinnaiyan *et al.*, 1997). The mammalian homologue of CED-4 has not been identified, but there is evidence for its existence (Chinnaiyan *et al.*, 1997).

CED-4 also binds to zymogens of cysteine proteases that contain large pro-regions. In this tripartite configuration, the cysteine protease is maintained as a zymogen (inactive). When the CED-4 interaction with Bcl-2 or Bcl-xL is disrupted, CED-4 interaction with the zymogen appears to be altered to induce autoproteolytic activation of the cysteine protease. FLICE/Machα1 is a member of the family of cysteine proteases that associate with CED-4. As in the TNFRI/Fas pathway, FLICE/Machα1 may be one of the earliest proteases activated during initiation of the proteolytic activation cascade.

The finding that the adapter protein CED-4 prevents zymogen activation when bound to Bcl-2 or Bcl-xL homodimers but activates zymogens when not bound likely explains the ability of overexpressed Bcl-2 or Bcl-xL to protect cells from apoptosis caused by numerous cellular signals or insults. When Bcl-2 or Bcl-xL homodimer levels are high, CED-4 is bound to the homodimers and is, therefore, unable to activate the cysteine protease cascade. This would also explain the anti-apoptotic activity of Bcl-2 and Bcl-xL truncated forms missing the mitochondrial targeting amino acid sequence (Borner *et al.*, 1994). As long as Bcl-2 or Bcl-xL can homodimerise, they have the potential to sequester all of CED-4.

Bcl-xL heterodimerization with the inducer family members, Bax, Bak or Bik, causes release of the CED-4-zymogen heterodimer from Bcl-xL (Chinnaiyan *et al.*, 1997). During or after dissociation from Bcl-xL, CED-4 induces the zymogen to undergo autoproteolytic activation. Thus, activation of cysteine proteases in this pathway directly depends on the dimerization state(s) of the Bcl-2 family members, and the dimerization state appears to be controlled by the availability of the apoptosis inducer members. The availability of inducer members for heterodimerization with Bcl-2 or Bcl-xL may be regulated by signals generated by the binding of cytokines.

Bad is a proapoptotic member of the Bcl-2 family and is sequestered in the cytosol when cytokines are present (Zha *et al.*, 1996). Sequestration occurs through the binding of Bad by a multifunctional protein, 14-3-3 (Zha *et al.*, 1996). This protein binds to phosphorylated proteins, and the Bad that is bound by 14-3-3 is phosphorylated on serine residues. Upon removal of cytokines, Bad becomes dephosphorylated at specific serine residues, dissociates from 14-3-3 and heterodimerizes with Bcl-xL. Bad phosphorylation and reassociation with 14-3-3 can be induced by addition of cytokines. Furthermore, hyper-phosphorylated Bad does not bind to Bcl-xL. These results suggest that the cytokine-engaged receptor prevents apoptosis by inducing the phosphorylation of the apoptotic-inducing members of the Bcl-2 family, thereby making them unavailable for dimerization with Bcl-2 or Bcl-xL.

The identity of the kinase pathway that couples cytokine receptor binding to phosphorylation of the pro-apoptotic Bcl-2 family members is under intense study. Activated PKB has been shown to prevent the apoptotic cell death of Rat-1 cells that are induced by c-*myc* (Kaufmann-Zeh *et al.*, 1997). Growth factor-dependent activation of PKB is through PI (3,4) P_2 generated by PI 3-kinase whose activation depends on the GTP-

binding protein Ras. PI 3-kinase is active when cytokines are bound to their receptors (see section 1.5). A potential role for the Ras activation of another kinase, Raf, has also been suggested (Wang *et al.*, 1996). Similarly, other phosphorylation events have been shown to affect apoptosis under experimental conditions (Nishina *et al.*, 1997 and Haldar *et al.*, 1995). The great advances in understanding the pathways that trigger apoptosis sets the stage for investigations into the regulation of the pathways by kinases and by levels of expression of proteins (e.g., the Bcl-2 family).

The suppressive signal appears to regulate the function of mitochondrial ion and/or water channels (Chao and Korsmeyer, 1998). Loss of the suppressive signal, when the anti-apoptotic cytokines are not available to the cell, leads to the failure of the channels to maintain the normal ion potential across the inner mitochondrial membrane and to the failure to maintain proper mitochondrial volume. Bcl-x and Bcl-2, are channel forming proteins responsible for maintaining normal mitochondrial physiology. Regulation of their activities is central to apoptosis (Chao and Korsmeyer, 1998).

Inhibition of Bcl activities leads to altered mitochondrial membrane permeability resulting in the release of cytochrome *c* into the cytosol. In the cytosol, cytochrome *c* is bound by the protein Apaf-1, an acronym for apoptotic protease-activating factor, which also binds caspase 9 and dATP. Binding of cytochrome *c* triggers activation of caspase 9, which then accelerates apoptosis by activating other caspases (Green and Reed, 1998).

The mechanism for cytochrome *c* release from the mitochondria is becoming clearer. Inhibition of normal mitochondrial channel function causes mitochondria to swell, rupture and release cytochrome *c* (Green and Kroemer, 1998). The inner mitochondrial membrane

has more surface area than the outer membrane. Swelling of the matrix extends the inner membrane, which then appears to cause rupture of the outer membrane, releasing cytochrome *c* from the inter-membrane space. This is consistent with the findings by many groups that the majority of cytochrome *c* in apoptotic cells is in the cytosol. Inhibitors of caspase activity did not prevent outer mitochondrial membrane rupture, indicating that the membrane rupture did not depend on caspase activity (Green and Kroemer, 1998).

An involvement of permeability transition pores in apoptosis has also been suggested (Green and Reed, 1998). These pores are multiprotein complexes that are present at sites where the inner mitochondrial membrane contacts the outer mitochondrial membrane. The relationship between the transition pores and members of the Bcl-2 family remain to be determined. Although the exact details require more investigation, it is clear that regulation of mitochondrial physiology by the Bcl-2 family and release of cytochrome c into the cytosol are critical events in the process of apoptosis that occurs in the absence of many viability-sustaining cytokines.

The Bcl-2 family may also be central to another type of apoptosis induction. It has long been recognised that the Bcl-2/Bcl-xL dimerisation state regulates the sensitivity of cells to death induced by free radicals (Hockenberry *et al.*, 1993 and Kane *et al.*, 1993). Free radical-induced cell death is accompanied by lipid peroxidation. Bcl-2 overexpression prevents free radical-induced lipid peroxidation. Cytochrome *c* is a one-electron carrier in mitochondrial electron transport. It is possible that cytosolic cytochrome c propagates or initiates free radical production. Although the mechanism for activation of apoptosis by these events is unknown, the findings suggest a function for the Bcl-2 family in regulating free radical damage. Bcl-xL forms pores in artificial membranes (Minn *et al.*, 1997) and has

a crystal structure similar to the β-subunit of diphtheria toxin (S. W. Muchmore, 1996). Diphtheria β-subunit translocates the α-subunit across membranes. Thus, Bcl-xL and Bcl-2 have the potential to translocate materials across membranes. Mitochondrial membrane pore formation and subsequent loss of mitochondrial transmembrane potential has been found to be one of the earliest cellular events associated with apoptosis (Castedd *et al.*, 1996). The molecular relationship between cytochrome *c*, the Bcl-2 family, activation of cysteine proteases, and free radicals remains to be determined.

Caspases – the executioners

While studying the develoment of the nematode *Caenorhabditis elegans*, two genes referred to as *ced-3* and *ced-4* were found to be essential for the programmed cell death which is crucial during ontogenesis of this simple organism (Yuan *et al.*, 1993). The *ced-4* gene encodes a polypeptide of unknown function whereas the *ced-3* gene encodes a protein, which exhibits a high similarity to a family of cysteine proteases which includes the interleukin-1-β-converting enzyme (ICE). These observations lead to the assumption that proteases like CED-3 and ICE are crucial for triggering the biochemical events, which lead to apoptotic cell death, in that they are responsible for the deliberate disassembly of the cell into apoptotic bodies during apoptosis (Thornberry and Lazebnik, 1998).

It is now well established that ICE is the prototype member of a family of related proteases. To unify the nomenclature of the ICE-like proteases, they are now referred to as caspases (cysteine proteases which cleave proteins after an aspartic acid residue). Caspases are present as inactive pro-enzymes that are activated by proteolytic cleavage. Caspases 8, 9 and 3 are situated at pivotal junctions in apoptosis pathways. Caspase 8 initiates disassembly in response to extracellular apoptosis-inducing ligands and is activated in a complex

associated with the cytoplasmic death domain of many cell surface receptors for the ligands (Thornberry and Lazebnik, 1998). Caspase 9 activates disassembly in response to agents or insults that trigger the release of cytochrome *c* from mitochondria and is activated when complexed with apoptotic protease activating factor 1 (APAF-1) and extra-mitochondrial cytochrome *c*. The Apaf-family of proteins in humans is homologue to the ced-family in C. elegans. Three genes, *ced-3*, *ced-4*, and *ced-9* encode the general apoptotic and anti-apoptotic program in *C. elegans*. Recently it has been shown that Apaf-3 turned out to be caspase-9, which has a similar structure to ced-3. Apaf-1 was the long sought mammalian homologue of ced-4. Apaf-1 needs a cofactor in order to bind to and therefore activate caspase-9. This cofactor was named Apaf-2 and seems to be identical with cytochrome *c* (Zou *et al.*, 1997 and Li *et al.*, 1997). Caspase 3 appears to amplify caspase 8 and caspase 9 initiation signals into full-fledged commitment to disassembly (Thornberry and Lazebnik, 1998 and Cryns and Yuan, 1998). Caspase 8 and caspase 9 activate caspase 3 by proteolytic cleavage and caspase 3 then cleaves vital cellular proteins or other caspases (Thornberry and Lazebnik, 1998 and Cryns and Yuan, 1998). Work with cells from caspase 3-/- and from caspase 9-/- mice suggests that the caspase pathway used for disassembly is cell-type specific (Hakem *et al.*, 1998; Kuida *et al.*, 1998; Kuida *et al.*, 1996 and Woo *et al.*, 1998). Embryonic stem cells (ESC), embryonic fibroblasts (EF), thymocytes, and splenocytes from caspase 3-/- and from caspase 9-/- mice were subjected to a wide array of agents or insults that induce apoptosis in cells from mice homozygous or heterozygous for the wild-type alleles. Whereas, ESC, EF, and thymocytes from caspase 9-/- mice were resistant to etoposide, splenocytes were sensitive. Caspase 9-/- ESC and EF were resistant to UV irradiation, however, thymocytes and splenocytes were sensitive. Cells from the caspase 3-/- mice showed a cell-type specificity for sensitivity to UV and γ irradiation.

The results suggest four possible caspase pathways. The first requires both caspase 3 and caspase 9. ESC from caspase 3-/- and from caspase 9-/- mice are resistant to UV irradiation and many other agents suggesting that this pathway involves cytochrome c release from mitochondria, caspase 9 activation, and subsequent cleavage of caspase 3 by caspase 9. The second pathway requires neither caspase 9 nor caspase 3. Thymocytes and splenocytes from caspase 3-/- and from caspase 9-/- mice are sensitive to UV irradiation suggesting the involvement of other caspases. The third requires caspase 9 but not caspase 3. Thymocytes from caspase 9-/- mice are resistant to γ-irradiation and dexamethasone, whereas thymocytes from caspase 3-/- mice are sensitive, suggesting that other caspases are directly activated by caspase 9. The fourth requires caspase 3 but not caspase 9. Activated splenocytes from caspase 3-/- mice are resistant to anti-CD95 whereas splenocytes from caspase 9-/- mice are sensitive. This pathway likely involves caspase 8 which then activates caspase 3.

Interpretation of the results is complicated by the existence of inhibitors that act by binding to caspases Deveraux *et al.*, 1997) or by competing against pro-caspases for binding to the protein complexes that activate caspases (Irmler *et al.*, 1998). Generation of the caspase 9 mutations involved substitution for a segment of caspase 9 required for enzymatic activity. The pro-domain that interacts with CARD (caspase recruitment domain) found in APAF-1 was left intact. Thus, mutant caspase 9s could compete with other caspases for binding to APAF-1.

The effects of caspase 3 and caspase 9 mutations *in vivo* are striking. Both cause perinatal mortality, and the most obvious effect is on brain development. Brain morphology in caspase 9-/- mice was more aberrant than in caspase 3-/- mice. Caspase 9-/- mice often

developed brain tissue outside the skull. This difference suggests that both caspase 9-, caspase 3-dependent and caspase 9-dependent, caspase 3-independent apoptosis pathways are involved in brain development. Altered brain morphology appeared to result, in part, from decreased apoptosis of neuroepithelial progenitors indicating that caspase 9 and caspase 3 are required to cull this population of cells.

Other gross morphological defects were not observed. Tissue remodelling involving retraction of the interdigital webbing in the foetal hand occurred normally in caspase 9-/- mice indicating that apoptosis during this developmental process is caspase 9-independent (Kuida *et al.*, 1998). Although the mutations confer resistance to thymocytes and splenocytes to many inducers of apoptosis, the mutation did not appear to affect thymic selection. Lymph nodes were, however, enlarged in the caspase 9-/- mice that survived 2 weeks.

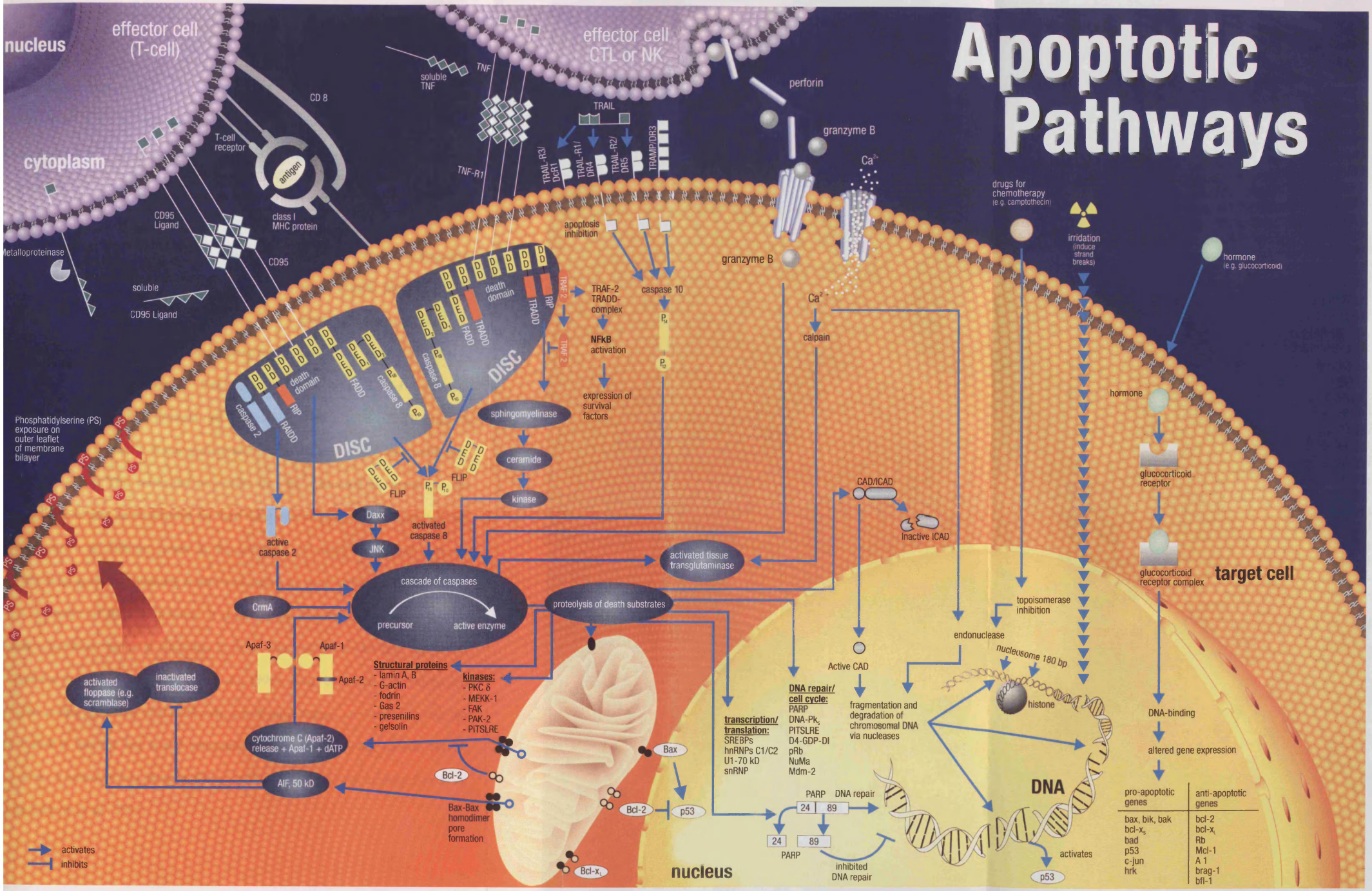
The caspases are probably the most important effector molecules, which induce apoptosis. The apoptotic signal transmitted by activated caspases can be downregulated by caspase inhibitors like the CrmA protein of poxviruses, the p35 protein of baculovirus or proteins encoded by the IAP (inhibitor of apoptosis) gene family that are endogenous mammalian apoptosis suppressers. However, it is thought that once Apoptosis Inducing Factor (AIF) and cytochrome *c* are released from the mitochondria during the apoptosis-induced permeability transition of the mitochondrial membrane, the activation of proenzymatic caspases and downstream events the point-of-no-return has been reached, after which the apoptotic events become irreversible. (Alnemri *et al.*, 1996; G. M. Cohen, 1997 and Nicholson and Thornberry, 1997).

DNA fragmentation - the end

One consequence of proteolysis of cellular proteins is the activation of Ca/Mg-dependent nucleases, especially DNAse I, which degrade the chromosomal DNA. This leads to multiple nicks and breaks within the DNA and finally results in the generation of DNA oligomers, their sizes being multiples of nucleosome-associated DNA. Thus, if the DNA from cells undergoing apoptosis is analysed by gel electrophoresis, a typical ladder pattern is observed which is a hallmark of apoptotic cell death (A. H. Wyllie, 1987). Recently it has been shown that during apoptosis a specific DNase, referred to as CAD, is activated. CAD and ICAD are complexed and caspase 3 cleavage releases the DNase activity, which causes DNA fragmentation in the nucleus (Enari *et al.*, 1998).

Many of the targets of activated caspases have already been characterised as being involved with the degradation of DNA. They include poly(ADP-ribose) polymerase (PARP) and DNA-dependent protein kinase (DNA-PKs) which are both involved in sensing DNA-damage and repair, U1 ribonucleoprotein, nuclear laminins, PKC-δ and cytoskeleton components like actin. PARP is one of the best examined targets of activated caspases. PARP is a DNA repair enzyme whose expression is triggered by DNA-strand breaks. In cells undergoing apoptosis PARP is cleaved from a 116 kD peptide into 24 kD and 89 kD polypeptides. It appears plausible that cleavage of PARP facilitates the degradation of cellular DNA, which is a hallmark of apoptosis (Lippke *et al.*, 1996 and De Murcia and De Murcia, 1994).

However, it is still unclear whether the caspases are directly responsible for apoptosis by destroying crucial cellular components or whether they activate further effector molecules. Probably both are true.


Apoptotic Pathways
nucleus
effector cell (T-cell)
cytoplasm
effector cell CTL or NK
soluble TNF
TNF
CD 8
T-cell receptor
antigen
class I MHC protein
CD95 Ligand
CD95
soluble CD95 Ligand
Metalloproteinase
TNF-R1
TRAIL
TRAIL-R3/ DCR1
TRAIL-R1/ DR4
TRAIL-R2/ DR5
TRAMP/DR3
perforin
granzyme B
Ca2+
drugs for chemotherapy (e.g. camptothecin)
irridation (induce strand breaks)
hormone (e.g. glucocorticoid)
apoptosis inhibition
granzyme B
Ca2+
calpain
TRAF-2 TRADD-complex
caspase 10
NFkB activation
expression of survival factors
death domain
TRADD
FADD
RIP
caspase 8
DISC
caspase 2
RAIDD
sphingomyelinase
ceramide
kinase
FLIP
Daxx
JNK
activated caspase 8
active caspase 2
Phosphatidylserine (PS) exposure on outer leaflet of membrane bilayer
activated tissue transglutaminase
CAD/ICAD
Inactive ICAD
cascade of caspases
precursor
active enzyme
CrmA
proteolysis of death substrates
topoisomerase inhibition
endonuclease
nucleosome 180 bp
histone
Active CAD
fragmentation and degradation of chromosomal DNA via nucleases
Apaf-3
Apaf-1
Apaf-2
Structural proteins
- lamin A, B
- G-actin
- fodrin
- Gas 2
- presenilins
- gelsolin
kinases:
- PKC δ
- MEKK-1
- FAK
- PAK-2
- PITSLRE
transcription/ translation:
SREBPs
hnRNPs C1/C2
U1-70 kD
snRNP
DNA repair/ cell cycle:
PARP
DNA-PKs
PITSLRE
D4-GDP-DI
pRb
NuMa
Mdm-2
activated floppase (e.g. scramblase)
inactivated translocase
cytochrome C (Apaf-2) release + Apaf-1 + dATP
AIF, 50 kD
Bcl-2
Bax
Bax-Bax homodimer pore formation
Bcl-2
p53
Bcl-xL
PARP
DNA repair
24
89
inhibited DNA repair
DNA
activates
p53
glucocorticoid receptor
glucocorticoid receptor complex
target cell
DNA-binding
altered gene expression
pro-apoptotic genes
bax, bik, bak
bcl-xs
bad
p53
c-jun
hrk
anti-apoptotic genes
bcl-2
bcl-xL
Rb
Mcl-1
A 1
brag-1
bfl-1
activates
inhibits

Apoptosis in colonic epithelium

As shown in figure 1.13, a wide array of proteins is involved in monitoring cellular proliferation and cell survival. In particular, there are two important factors that relate to intestinal apoptosis:

(i) Differential expression of Bcl-2 may regulate susceptibility to epithelial apoptosis and colon carcinoma. Stem cells at the base of the colonic crypts express higher levels of Bcl-2 than those of the small intestine and therefore have a greater chance of survival in a background of genomic damage, which may progress to cancer (C. S. Potten, 1997). However, another Bcl-2 family member, Bak, has also been shown to inhibit the anti-apoptotic effect of Bcl-2 and lead to rapid and extensive apoptosis. Whilst Bak's function has been likened to that of Bax, it shares greater sequence homology with Bcl-2 and Bcl-x_L Chittenden *et al.*, 1995). This raises the possibility that the structural elements that are shared by Bak and Bax may be responsible for their functional similarity. It is suggested that Bak and Bax operate at different points in the apoptosis pathway, where Bak may act directly on one of its apoptosis-inducing relatives to moderate their action whilst Bax might interact with a number of survival proteins. It is interesting to note that increased colonic epithelial Bak expression results in apoptosis in these cells and decreased Bak expression has been observed in colon neoplasia (Moss *et al.*, 1996). Also, increased Bak expression has been found during apoptosis of HT-29 cells, which have mutant p53, implying that the regulation of Bak in this cell line is p53-independent (Moss *et al.*, 1996). Indeed, HT-29 cells also express a constitutively phosphorylated form of Bcl-2, perhaps keeping Bak in an inactive state (Merritt *et al.*, 1995).

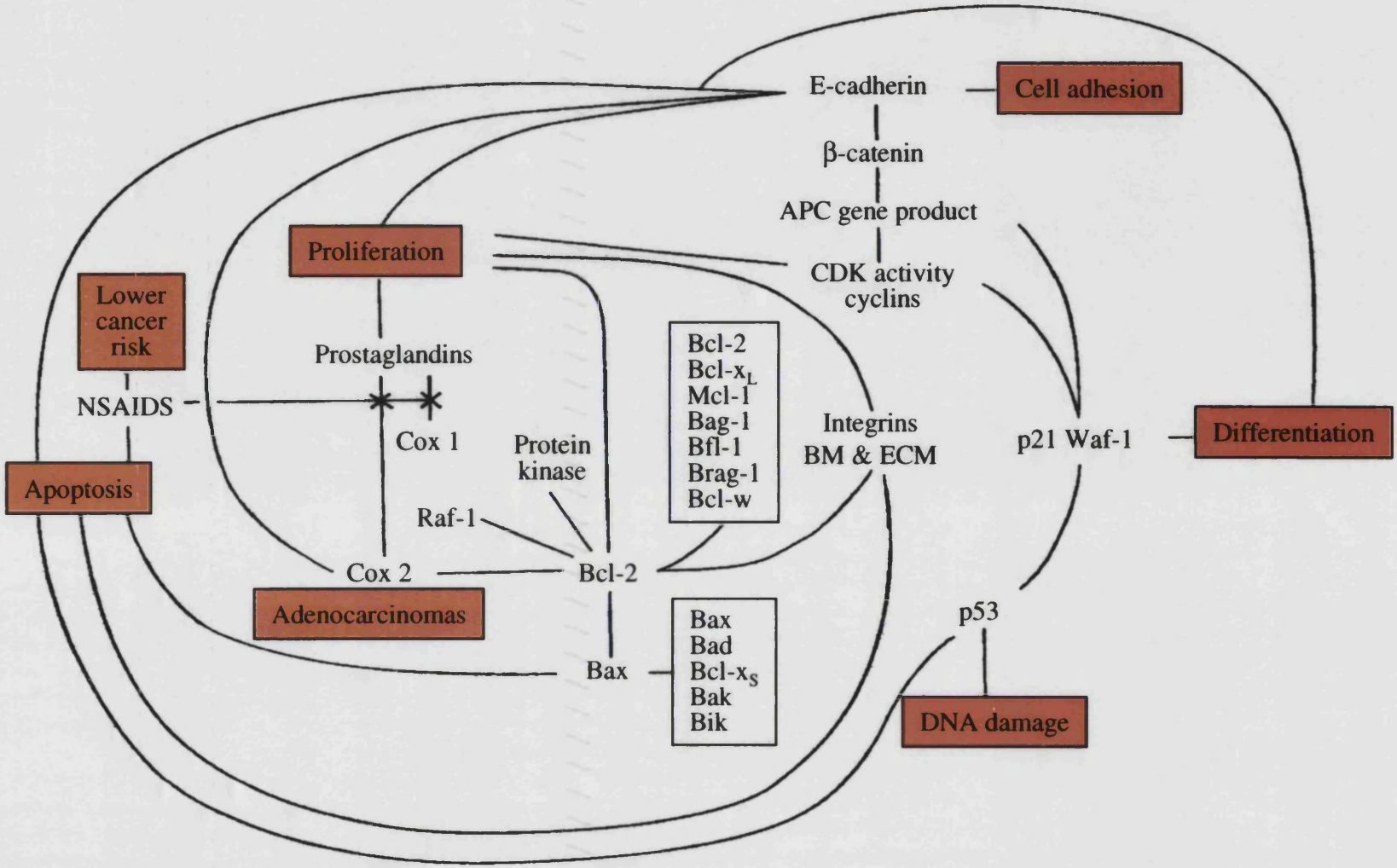


Figure 1.15. Molecular and biochemical interactions that have been proposed to determine levels of apoptosis in the gastrointestinal tract. Lines indicate probable interactions that regulate events shown in shaded boxes. Links include physical association of proteins, as well as upregulation or downregulation of expression or activity. Most linkages are not intended to display a positive or negative interaction because of the tentative nature of current understanding of such events, with the exception of tha ability of nonsteroidal anti-inflammatory drugs (NSAIDS) to inhibit cyclooxygenase enzymes. Adapted from Potten, 1997.

(ii) Cell-cell interactions are also required to maintain epithelial survival. Most epithelia are cohesive tissues where the cells interact with each other as well as the basement membrane. It now appears that adhesion through cell-cell interactions contributes to the cohesive integrity of epithelia. A colon carcinoma cell line (LIM 1863), cultured as structural organoids containing polarised columnar and goblet cells, can be reversibly disaggregated in the absence of calcium. When cell-cell adhesion during the reaggregation process is prevented by a function-blocking anti-αv integrin antibody, however, the intestinal cells enter an apoptosis programme termed anoikis (Bates *et al.*, 1994 and Frisch and Francis, 1994). Cell-cell adhesion through the cadherin system has been implicated directly for epithelial cell survival *in vivo* (Metcalfe and Streuli, 1997). Cadherin-mediated adhesion provides a mechanistic basis for regulating epithelial integrity. Thus, survival signals from both cell-cell and cell-matrix adhesion molecules cooperate to ensure that cells with regional specialisation maintain their positional identity and do not migrate into innappropriate places.

When examined within the framework of the cell biology of the gastrointestinal tract and, taking into account the other genes known to be involved, genetic manipulations that result in gene deletions or overexpression offer the imminent prospect of a better understanding of how apoptosis is regulated and how apoptosis interacts with proliferation.

1.7 AIMS

This study is based on the hypothesis that the intestinal epithelium constitutes an interface between the host and the environment and plays a crucial role as an outpost of the immune system located in the underlying gut mucosa. This is an exploration into their involvement in immune and inflammatory reactions under the control of immune and inflammatory mediators, such as cytokines and nitric oxide. The main objectives of the investigation were:

1. To investigate the intracellular signalling mechanisms involved in nitric oxide production and regulation in human colonic epithelial cells by pro- and anti-inflammatory cytokines.

2. To examine the role of the intracellular signalling enzyme, PI 3-kinase, on iNOS regulation, via IL-13.

3. To establish the profile of pro-inflammatory cytokines which induce apoptosis in human colonic epithelial cells and to characterise whether IL-13 and/or NO play any part in this process.

4. To examine the role of the PI 3-kinase pathway and some of its downstream targets on the apoptotic pathway induced in these cells.

The elucidation of the role of colonic epithelium in the inflammatory process and the mechanisms thereof could offer a novel approach in the development of new diagnostic tests and potential new therapeutic strategies for the effective treatment of intestinal inflammation.

2. MATERIALS AND METHODS

2.1 MATERIALS

Material	Source
β-Glycerophosphate	Sigma, Poole (UK)
α-Human Fas mAb (IgM CH-11 clone)	Upstate Biotechnology (TCS Biologicals, Buckingham, UK).
[γ-^{32}P]ATP (3000 Ci/mmol)	DuPont NEN (Stevenage, UK)
[^{32}P]-Orthophosphate (8500-9120 Ci/mmol)	DuPont NEN (Stevenage, UK)
2,3-Diaminonaphthalene (DAN)	Lancaster Synthesis Ltd.
2-Mercaptoethanol	Sigma, Poole (UK)
3-[N-morpholino]-propane-sulfonic acid (MOPS)	Sigma, Poole (UK)
4G10, monoclonal Ab	Upstate Biotechnology, USA
5'Digoxygenin labelled iNOS probe	R&D Systems (Abingdon,UK)
Absolute Ethanol	Hayman Ltd., Witham, UK
Acrylamide/bis acrylamide	Bio-Rad, UK
Adenosine triphosphate	Sigma, Poole (UK)
Agarose	Sigma, Poole (UK)
Ammonium persulphate	BDH, Poole (UK)
Annexin-V-Fluos	Boehringer Mannheim (UK)
Apoptag Direct	Oncor (Appligene, Durham, UK)
Apoptosis Detection Kit	(R&D Systems, Abingdon, UK)
Bad, monoclonal Ab	Transduction Laboratories, USA
Bad, polyclonal Ab	Santa Cruz, USA
Bak polyclonal Ab	Upstate Biotechnology, USA
Bcl-2, monoclonal Ab	Upstate Biotechnology, USA
Bcl-$x_{L,}$ polyclonal Ab	Transduction Laboratories, USA
Blocking reagent (for Northern analysis)	Boehringer Mannheim (UK)
Bovine serum albumin (BSA)	Sigma, Poole (UK)
Bromophenol blue	BDH, Poole (UK)
Cell culture plastics	Nunc, UK
Chloroform	Fisons, Loughborough (UK)
Coomassie blue	Sigma, Poole (UK)
Cycloheximide	Sigma, Poole (UK)
Diethyl pyrocarbonate (DEPC)	Sigma, Poole (UK)
Digoxigenin chemiluminescent detection kit for Northern blotting	Boehringer Mannheim (UK)
Dimethyl sulphoxide (DMSO)	Sigma, Poole (UK)
Dithiothretol (DTT)	Sigma, Poole (UK)
DOTAP	Boehringer Mannheim (UK)
ELISA^{PLUS}	Boehringer Mannheim (UK)

Enhanced chemiluminescence detection kit for Western blotting (ECL)	Amersham International, UK
Ethidium bromide	Sigma, Poole (UK)
Ethylenediaminetetraacetic acid (EDTA)	Sigma, Poole (UK)
Flo-scint IV scintillation fluid	Canberra Packard (UK)
Foetal bovine serum (FBS)	Gibco BRL, Paisley, UK
Folch lipids	Sigma, Poole (UK)
Formaldehyde	BDH, Poole (UK)
Formamide	BDH, Poole (UK)
Glacial acetic acid	Fisons, Loughborough (UK)
Glycerol	Sigma, Poole (UK)
Glycine	Sigma, Poole (UK)
Goat anti-mouse peroxidase conjugate	DAKO, Denmark
Goat anti-rabbit peroxidase conjugate	DAKO, Denmark
Hank's balanced salt solution	Gibco BRL, Paisley, UK
Hepes (1M liquid)	Gibco BRL, Paisley, UK
Histone H2B	Boehringer Mannheim, UK. Stock stored at -20°C at 1mg/ml in 20mM Tris pH 7.5
Hydrochloric acid	BDH, Poole (UK)
IFN-γ: human recombinant; specific activity > 2×10^7 U/mg	Boehringer Mannheim, UK; stored in aliquots at -70°C
IL-1α: human recombinant; specific activity 5×10^7 U/mg	Gift from Glaxo (Greenford, UK); diluted in sterile PBS + 0.25% (w/v) BSA and stored in aliquots at -70°C)
IL-13: purified from culture supernatants of stable transfected CHO cells (Minty *et al.*, 1993)	Gift from Dr. A. Minty (Sanofi Recherche, Labege, France); diluted in sterile PBS + 0.25% (w/v) BSA and stored in aliquots at -70°C
IL-4: human recombinant; specific activity > 1×10^7 U/mg	Genzyme
Insulin	Donated by Dr. M. Welham
Iodine	Sigma, Poole (UK)
IRS-1, polyclonal Ab	Upstate Biotechnology, USA
IRS-2, polyclonal Ab	Upstate Biotechnology, USA
Leupeptin	Sigma, Poole (UK)
Lithium chloride (LiCl)	Sigma, Poole (UK)
LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one)	Affiniti (Exeter, UK)
Maleic acid	Sigma, Poole (UK)
McCoy's 5A medium	Gibco BRL, Paisley, UK
Methanol	Fisons, Loughborough (UK)
Methylamine	Fisons, Loughborough (UK)
Molecular weight markers	Bio-Rad
Neutral buffered formalin	Sigma, Poole (UK)
Nitrocellulose	(Schleicher and Schuell)

NP40	Sigma, Poole (UK)
Ovalbumin	Donated by Dr. M. Welham
p70S6K, polyclonal Ab	New England Biolabs, USA
p70S6K ^{411}Ser, polyclonal Ab	New England Biolabs, USA
p70S6K ^{421}Thr/^{424}Ser, polyclonal Ab	New England Biolabs, USA
p85 (SH3/bcr), polyclonal Ab	Dr. M. Welham, Bath, UK
p85α, monoclonal Ab	Dr. D. Cantrell, I.C.R.F., UK
Phosphate buffered saline	Gibco BRL, Paisley, UK
Phosphatidylinositol	Sigma, Poole (UK)
Phosphatidylserine	Sigma, Poole (UK)
Phosphotyrosine antibody (4G10)	Gift from Dr. M. Welham
PKB-α ^{473}Ser, polyclonal Ab	New England Biolabs, USA
PKBα polyclonal Ab	Brian Hemmings (Friedrich Miescher-Institute, Switzerland)
PKB-α, polyclonal Ab	Santa Cruz, USA
PKB-α, polyclonal Ab	New England Biolabs, USA
PMSF	Sigma, Poole (UK)
Polaroid film (type 55)	Sigma, Poole (UK)
Ponceau S	Sigma, Poole (UK)
Potassium oxalate	Sigma, Poole (UK)
Propan-1-ol	Fisons, Loughborough (UK)
Propan-2-ol	Fisons, Loughborough (UK)
Propidium iodide	Sigma, Poole (UK)
Protease Assay Kits	Chemicon, USA
Protein A beads	Pharmacia, UK
Protein G beads	Sigma, Poole (UK)
Protein kinase inhibitor (PKI)	Sigma, Poole (UK). Stored at -20°C as 5μM stock diluted in 0.05% BSA
RNase A	Sigma, Poole (UK)
RNAzol B	Tel Test, Texas, USA
Sarcosyl	BDH, Poole (UK)
Shc, polyclonal Ab	Upstate Biotechnology, USA
SHIP/SH2-GST fusion protein	Mark Coggeshall, Ohio State University, USA
SHPTP2, polyclonal Ab	Santa Cruz, USA
Sodium azide	Sigma, Poole (UK)
Sodium chloride	Sigma, Poole (UK)
Sodium dodecyl sulfate (SDS)	Sigma, Poole (UK)
Sodium fluoride	Sigma, Poole (UK)
Sodium hydroxide	Sigma, Poole (UK)
Sodium molybdate	Sigma, Poole (UK)
Sodium nitrite	Sigma, Poole (UK)
Sodium orthovanadate	Sigma, Poole (UK)
TEMED	Sigma, Poole (UK)

Tetrabutylammoniumhydrogen sulphate	Fluka, Germany
Tissue culture reagents	Gibco BRL, Paisley, UK
TNF-α: human recombinant; specific activity 6×10^7 U/mg	Gift from Bayer (Slough, UK); diluted in sterile PBS + 0.1% (w/v) BSA and stored in aliquots at-70°C)
Trisodium citrate dihydrate	Sigma, Poole (UK)
Triton X-100	Sigma, Poole (UK)
Trizma base	Sigma, Poole (UK)
Tween-20	Sigma, Poole (UK)
Versene	Gibco BRL, Paisley, UK
Wortmannin	Sigma, Poole (UK)
X-OMAT film	Amersham International, UK
Z-VAD-FMK	Calbiochem (Nottingham, UK)

2.2 METHODS

2.2.1 Cell Culture

The HT-29 colon adenocarcinoma cell line is a grade II human epithelial cell line that was isolated from a primary tumour in a 44 year old Caucasian female (ECACC). They are well characterised with features that match normal intestinal epithelium, such as epithelial polarity, presence of the actin-binding protein villin and the occurrence of an enterocytic differentiation (Chantret *et al.*, 1988).

HT-29 cells were routinely cultured in 80cm^2 tissue culture flasks in McCoy's medium supplemented with penicillin (10 units/ml), streptomycin (10 µg/ml), fungizone (0.5µg/ml) and 10% (v/v) foetal bovine serum (FBS) (referred to as complete medium). Cultures were maintained at 37°C in an atmosphere of 5% CO_2. The medium was changed every 3 days. To subculture confluent monolayers, the medium was removed and the cells were washed 3x with PBS (w/o Ca^{2+} and Mg^{2+}). Cells were then washed once with a 3ml Trypsin-EDTA mixture of 0.05% (w/v) Trypsin and 0.02% (w/v) EDTA. The excess solution was removed and the cells were incubated for approximately 5 min at 37°C until the cells had detached from the flask. Adding 10ml of complete medium inhibited the action of Trypsin-EDTA and the cell suspension was centrifuged at 200xg for 5 min. The cell pellet was resuspended in complete medium and cell counting and viability were checked in a Neubauer haemocytometer after mixing with Trypan Blue. Dead cells stained blue, due to the uptake of Trypan Blue. Cell viability was always greater than 95%. Cells were counted and then seeded at 2-3 x 10^4/ml of McCoy's complete medium, into 80cm^2 tissue culture flasks for further culture, or into 6-, 24- or 96- well plates or Petri dishes for experimental protocols.

For storage, cells were resuspended at 4 x 10^6 cells/ml of freeze medium (Buffersand Solutions 2.3.1). The cell suspension was transferred to cryotubes at 1 ml/tube, gradually cooled in vapour phase of liquid nitrogen overnight and tubes were stored in liquid nitrogen tanks. For resuscitation of cells from liquid nitrogen, cells were rapidly defrosted at 37°C in a water bath, washed in McCoy's medium, resuspended in complete medium and cells from one cryotube were seeded into 80cm^2 tissue culture flasks in McCoy's medium, continuing as above.

2.2.2 Experimental protocol

Unless otherwise stated, HT-29 cells were grown until confluent. Prior to experiments, monolayers were washed and cultured in McCoy's without FBS for 24 hours. Growth-arrested cultures were treated with fresh FBS-free medium and stimulated with the appropriate doses of either drugs, cytokines or vehicle controls for the times described in the results section. Supernatants were collected, centrifuged to remove cellular debris and stored at -70°C until assayed for extracellular nitrite (see below). Total RNA and cellular proteins were extracted as described below.

2.2.3 Fluorometric nitrite assay

Nitric oxide (NO) production by HT-29 cells was determined by measuring the stable end-product nitrite in the cell culture supernatants by a fluorometric assay which is based upon the reaction of nitrite, under acidic conditions, with 2,3-diaminonaphthalene (DAN) to form the fluorescent product 1-(H)- naphthotriazole. The assay was modified for use on a Photon Technology International (PTI) spectrofluorimeter from the method previously described in (Misko *et al.*, 1993). 200 µl of freshly prepared DAN (0.05 mg/ml in 0.62M HCl) was added to 2 ml of sample (culture supernatants) and vortexed immediately. After 10'

incubation at room temperature in the dark, the reaction was terminated with 100 µl of 2.8N NaOH. The samples were measured using a PTI dual wavelength spectrofluorimeter ($\lambda_{excitation}$365nm and $\lambda_{emission}$405nm) and compared with known concentrations of sodium nitrite (10nM-2µM sodium nitrite in FBS-free McCoy's medium). Phenol red present in McCoy's medium did not interfere with the assay. The sensitivity of the assay is 10 nM.

2.2.4 Northern analysis

Total cellular RNA was extracted from HT-29 cells using a commercial solution containing guanidinium thiocyanate (RNAzol B), a modification of the method described by {Chomczynski and Sacchi, 1987}. Buffers used for Northern analysis are detailed below and all solvents were "HPLC" grade.

2.2.4.1 RNA isolation

Monolayers were lysed directly in the culture dish by the addition of 1ml RNAzol B per well ($3.5x10^6$ cells). The lysate was homogenised with a sterile cell scraper and transferred to sterile Eppendorf tubes by pipette. The addition of 100µl chloroform to homogenates on ice for 5' was followed by centrifugation at 12 000xg (4°C) for 15'. The homogenate forms two phases and the upper colourless aqueous phase, containing the RNA, is transferred to a fresh sterile tube. An equal volume of isopropanol was added and kept on ice for 15 minutes. After centrifugation at 12 000xg (4°C) for 15', the RNA precipitate forms a white pellet at the bottom of the tube.

2.2.4.2 Sample preparation

The RNA pellet was washed with 1ml of ice-cold 75% ethanol. The pellet was dried, resuspended in sterile RNase-free Milli-Q water and the concentration measured by absorbance of 2µl RNA in 1 ml 0.1 M NaOH at 260 nm. The amount of RNA (in µg) present in each sample was calculated by:

A_{260} x dilution factor (500) x 40 x volume of remaining RNA solution in ml (0.048)

ODs were also read at 280 nm and 230 nm to assess the purity of RNA. A value of less than 2 for the OD_{260} : OD_{280} ratio indicated protein contamination. A low OD_{260} : OD_{230} ratio indicated guanidine contamination. 30 µl of RNA sample buffer was added to 10µg RNA and the samples were vortexed and heated for 15-30' at 80°C. The samples were cooled on ice and 2.5 µl of bromophenol blue was added. Samples were mixed and pulse centrifuged prior to loading on agarose gels.

2.2.4.3 Gel preparation and transblotting

1 % agarose gel was prepared by dissolving 3 g agarose in 230 ml DEPC-treated water using a microwave oven. 15 ml 20X MOPS buffer and 54 ml formaldehyde were added and the gel solution allowed to cool to ~60°C before pouring. The gel was set with two 15-lane combs using tanks purchased from Hoefer Scientific Instruments (Newcastle, UK). After 40', the gel was transferred to a submarine tank (Hoefer), which was surrounded with ice and covered with cold 1X MOPS running buffer. 10 µg RNA per lane was loaded and the gel run at constant current of 100 mA, until the bromophenol blue band had migrated 1.3 inches (~2 hours). The gel was placed under a UV light and the ethidium bromide stained 18S and 28S ribosomal RNA bands observed to assess equal loading. The gel was photographed using a polaroid CU5 88-46 land camera (Genetic Research Instrumentation Ltd.) and type 55 polaroid film. The gel was agitated gently in DEPC-treated water for 30-

60' to remove formaldehyde, prior to transblotting. The blotting tank consisted of a glass plate suspended in a sandwich box, which was half-filled with 20X SSC buffer. A wide strip of filter paper placed over the glass plate and reaching down into the buffer solution at each end acted as a wick. The gel was placed upside down on the filter paper and covered with a piece of positively charged nylon membrane with 3 pieces of similar size paper and a stack of paper towels. A 500 g weight was placed on top and left overnight to allow the RNA to transfer by capillary action. The RNA was fixed onto the nylon membrane by baking in a vacuum oven (Jouan) at 120°C for 20'. The membrane was sealed in a plastic bag and stored at room temperature prior to hybridisation.

2.2.4.4 Hybridisation with DIG-labelled oligonucleotides

Hybridisation of membranes and detection of bound probes was performed essentially as described in the Digoxigenin (DIG) chemiluminescent detection kit for Northern blotting from Boehringer Mannheim. The DIG detection system is based on the labelling of nucleic acid probes with a steroid hapten, digoxigenin. The DIG-labelled probes are hybridised to membrane-bound RNA. Specific hybridisation is immuno-detected with an alkaline phosphatase conjugated anti-digoxigenin antibody and visualised with the chemiluminescent substrate, CSPD, using X-ray film. Hybridisation temperature was optimised for the iNOS probe at 60°C, at a final concentration of 10 ng/ml. The volumes specified are for a 100 cm^2 membrane and prehybridisation, hybridisation and wash steps were all performed at 60°C. The membrane was prehybridised by incubating with 20 ml hybridisation solution for 1 hour in a sealed bag. The hybridisation solution was discarded and 2.5 ml of probe diluted to 10 ng/ml in hybridisation solution was added. All air bubbles were removed and the bag resealed and incubated overnight. The membrane was transferred to a small sandwich box and washed twice for 5' each in 2X SSC, 0.1% SDS solution, followed by two washes of 5'

each in 0.1X SSC, 0.1% SDS solution at 60°C. The following steps were performed at room temperature on a shaking platform. Membranes were washed for 5' in wash buffer, prior to blocking for 30' in 100 ml buffer 2. Membranes were incubated for 30' with 20 ml alkaline phosphatase conjugated anti-DIG antibody diluted 1:10 000 in buffer 2. Membranes were then washed twice for 15' each in wash buffer and equilibrated for 2-5' in buffer 3. The membrane was drained and incubated for 5' between 2 plastic sheets with 1 ml of lumigen PPD substrate diluted 1:100 in buffer 3. The membrane was drained again, sealed in a plastic bag and incubated for 15' at 37°C in the dark, followed by exposure to Kodak X-OMAT X-ray film for 4-6 h at room temperature and the film developed using an RGII Fuji X-ray film developer.

2.2.5 Western blot analysis

2.2.5.1 Cell lysis and sample preparation

Monolayers of HT-29 cells (10^7 cells/ Petri dish) were stimulated and incubated at 37°C in McCoy's as described above. Stimulations were terminated at the appropriate times by aspiration of the supernatant and the addition of 1 ml ice-cold lysis buffer (Buffers and Solutions 2.3.4). Cells were solubilised using a cell scraper and the resulting lysates transferred to Eppendorf tubes which were incubated at 4°C for 15', followed by centrifugation at 12 000xg for 2' at 4°C. Supernatants were transferred to fresh Eppendorf tubes and kept on ice.

2.2.5.2 Protein assay

Total protein per lysate was estimated using the Bio-Rad *DC* Protein Assay. This assay is based on the Bradford dye-binding procedure (Bradford, 1976). Known concentrations of

bovine serum albumin (BSA) diluted in lysis buffer were used as a standard curve. 5µl of sample or standard were placed in a 96-well plate with 25 µl of working reagent A' (20µl reagent S into 1ml reagent A), plus 200µl of Bio-Rad reagent B, provided in the kit. After 15', the plate was read at 595 nm on a Dynatech MR5000 platereader. The protein concentrations were calculated by linear regression from the standard curve and, if significantly variable, the lysate volumes were adjusted using lysis buffer, thus ensuring equal concentrations of protein in each sample.

2.2.5.3 Whole cell extracts

An aliquot, usually 20µl, of lysate was removed, placed into a tube containing 5X SDS sample buffer, boiled for 2-5' and pulse spun before loading onto a gel.

2.2.5.4 Immunoprecipitation

The remaining lysate was precleared by adding 20µl of a 50% suspension (v/v) of either protein-A or protein-G sepharose beads (depending on the antibody to be used) and rotated for 15-30' at 4°C. The beads were spun down at 12000xg for 1' and the supernatant removed to a clean tube. The appropriate antibody was then added to the precleared extract, briefly vortexed and incubated on ice for 30' - 2h. After addition of a 50% (v/v) suspension of the appropriate protein sepharose beads, the samples were rotated for 30 - 60' at 4°C. The beads were captured by centrifugation at 12000xg for 1' at 4°C. When appropriate an aliquot (usually 20 µl) of each sample was removed to a tube containing 5X SDS sample buffer, boiled for 2-5' and pulse spun before loading the gel.

The remaining supernatant was aspirated and the pellet washed 3X in 1ml of lysis buffer, with the immunocomplexes spun down between washes. After the last wash, the

supernatant was aspirated and any surplus supernatant was removed with a Hamilton syringe. The pellet was resuspended in 20 – 40 µl of 1X SDS sample buffer, boiled for 2' and pulse spun before loading the gel.

2.2.5.5 Separation of cellular proteins by electrophoresis

Proteins were analysed by one dimensional gel electrophoresis, which under reducing conditions separates proteins based on molecule size. Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out essentially as described by Laemmli (Laemmli, 1976). Proteins were separated by SDS-PAGE using the Bio-Rad Mini Protean II.

Minigels of the appropriate percentage were prepared as described in buffers and solutions. The resolving gel was poured into the gel equipment and overlaid with Milli-Q water. Polymerisation took 20 - 30', after which the water was aspirated off, the stacking gel was poured and a 10 or 15 lane comb inserted. Polymerisation took 20', the comb was removed and the wells washed thoroughly with Milli-Q water. The wells were then filled with 1X SDS-PAGE running buffer.

20 µl of each sample was then loaded into the wells in parallel with molecular weight markers and the gels run at 80 V through the stacking gel, followed by 150 V through the resolving gel, until the bromophenol blue reached the bottom of the gel. Gels were then placed in transfer buffer.

2.2.5.6 Semi-dry transfer of proteins to nitrocellulose

The graphite electrodes of the semi-dry transfer apparatus (Pharmacia-Biotech Multiphor II) were dampened with semi-dry transfer buffer, followed by placing a sandwich of 4 pieces of 3MM Whatmann paper (the same size as the gel), one piece of nitrocellulose membrane, the gel and another 4 pieces of 3MM paper, all soaked in transfer buffer. Each layer was rolled gently to expel air bubbles. The transfer was run for 60' at 0.8 mA/cm^2 of membrane. The membrane was then stained with Ponceau S to check for transfer and even loading of the samples and to determine the location of the molecular weight markers. The stain was removed by washing the membrane in distilled water for 2', followed by a 10' wash in Tris buffered saline (TBS).

2.2.5.7 Blocking and Developing

The non-specific protein binding was blocked by overnight incubation of the membrane with the appropriate blocking buffer at room temperature on a rocking platform. After a 10' wash in TBS, the membrane was incubated with the primary antibody diluted in a 1:5 dilution of fresh blocking buffer for 2h – overnight (usually 3h). Membranes were washed 1X with TBS, 3X with TBSN, 1X with TBS for ~10' each wash. The membrane was incubated for 1 – 2h with the appropriate secondary antibody diluted in TBSN (0.1 μg/ml), followed by extensive washing as described above. An extra TBS wash for 10' was done before adding 10 ml of Enhanced Chemiluminescent (ECL) reagent for 1'. The membrane was exposed to X-ray film for 30" - 30' and the film was developed using an RGII Fuji X-ray film developer.

2.2.5.8 Membrane stripping

Where appropriate, blots were stripped of bound protein and reprobed with a different primary antibody. After the ECL procedure described above, the membrane would be

washed once in TBS for 10', placed in 50 ml of stripping buffer in a sealed sandwich box and incubated for 1 hour at 55°C. After extensive washing in at least three changes of TBSN and one wash in TBS, the membrane would be reblocked for 1 hour in blocking buffer. A different primary antibody could then be applied to the membrane for further protein detection.

2.2.6 Measurement of $[Ca^{2+}]_i$

The measurement of $[Ca^{2+}]_i$ has been made possible by the development of a number of calcium sensitive dyes including fura-2 (review Walt *et al.*, 1987). Fura-2 can be non-disruptively loaded into cells as the acetoxymethyl ester (AM), since the esterified fura-2 is uncharged and hydrophobic and can cross the plasma membrane. Once in the cells, endogenous esterases release the free fura-2, which cannot permeate out of the cell. The fluorescence excitation maximum of fura-2 shifts to a lower wavelength upon calcium binding, without any change in the emission maximum. Thus, fura-2 can be used as a dual excitation indicator. The excitation maxima for calcium-free and calcium-bound fura-2 are monitored at 380nm and 340nm respectively. The emission maximum is monitored at 510nm.

2.2.6.1 Loading cells with fura-2/AM

HT-29 cells were grown on 22mm diameter glass cover slips. When subconfluent, the cells were washed twice in HBSS, and then loaded with 5μM fura-2/AM at 37°C for 40' in complete HBSS. The adherent cells were then washed twice and placed in a 37°C chamber with HBSS.

2.2.6.2 $[Ca^{2+}]_i$ measurements

The chamber was positioned on a fluorescence microscope from Photon Technology International and a population of approximately 30 cells was selected by adjusting the field of view. Using a X40 oil immersion objective, the fluorescence changes were monitored over 40" for a basal intracellular calcium measurement. After addition of the agonist, the response was monitored for at least 1' and detected using dual excitation wavelengths of 340nm and 380nm and a single emission of 510nm on a dual excitation/dual emission spectrofluorimeter from Photon Technology International. Due to the fact that when using this method, the fluorimeter can not be calibrated, the results were expressed as the fluorescence ratio at 340/380nm wavelengths.

2.2.7 PI 3-kinase activity

Two separate methods for measuring the agonist-induced activation of PI 3-kinase were employed in this study. The first method involves the measurement of the accumulation of D-3 phosphatidylinositol lipids in intact cells whereas the second method relies on the immunoprecipitation of the Class 1A PI 3-kinase enzyme using an antibody to the p85 subunit, followed by an *in vitro* lipid kinase assay to analyse the immunoprecipitates. These two assays differ in the type of PI 3-kinase activity they measure. The former accounts for all PI 3-kinase activity that arises within the cells, whereas the latter only measures the activity of the PTK/SH2-coupled PI 3-kinase, which is immunoprecipitated by the p85 antibody.

2.2.7.1 Accumulation of D-3 phosphatidylinositol lipids in intact cells

2.2.7.1.1 Sample preparation

HT-29 cells were dissociated from the flask using 3ml of Versene, washed three times in phosphate-free Dulbecco's Modified Eagles medium (DMEM) with 10-minute incubations at 37°C before each spin of 400xg for 5'. The cells were subsequently resuspended in 10ml phosphate-free DMEM containing 10% dialysed foetal bovine serum and 20mM HEPES at 10^7 cells/ml and incubated at 37°C for 4 h with 1mCi of [^{32}P]-orthophosphoric acid (100μCi/ml, 185MBq).

After incubation, the cells were washed three times in phosphate-free DMEM and resuspended in McCoy's (with 20mM HEPES, but without FBS). 10^7 [^{32}P]-labelled cells were aliquoted in a volume of 120μl into 1.5ml Eppendorf tubes and equilibrated at 37°C for 10'. Each point was stimulated with 12μl of agonist or vehicle for the appropriate times and the reaction quenched by the addition of 700μl of ice cold chloroform/methanol/water (32.6%/65.3%/2.1%) according to the method described by (Jackson *et al.*, 1992). The samples were immediately placed on ice. 600μl of chloroform containing 10μg of Folch lipids as a carrier protein were then added along with 100μl of 2.4M acid (HCl), 5mM tetrabutylammoniumhydrogen sulphate. The extraction mixtures were vortexed and centrifuged at 3000xg for 5'. The lower phase was removed and added to 400μl of 1M HCl, 5mM EDTA. The mixtures were again vortexed and centrifuged at 3000xg for 5'. The lower phase was removed and dried *in vacuo* using a Savant SpeediVac. When dried down, 1ml of 25% (w/v) methylamine/methanol/N-butanol (4/4/1) was added to the residue and, after vortexing, the samples were incubated in a 53°C water bath for 40'. This deacylation procedure renders the glycerophosphorylinositol derivatives of PI (3) *P* (GroPI (3) *P*), PI

(3,4) P_2 (GroPI (3,4) P_2) and PI (3,4,5) P_3 (GroPI (3,4,5) P_3) water soluble. The samples were then cooled for 1' on ice and dried *in vacuo*. Finally, 500µl of water and 600µl of N-butanol/40-60% petroleum ether/ethyl formate (20/4/1) were added. The samples were vortexed and centrifuged at 750xg for 30". The upper phase was removed and discarded and the lower phase was dried *in vacuo*. The samples were then stored at -20°C until analysis.

2.2.7.1.2 HPLC analysis of samples

Anion exchange high performance liquid chromatography (HPLC) was used to analyse the lipid content of the samples using a water and phosphate buffer gradient (Stephens *et al.*, 1989). The samples were resuspended in water and injected onto a Partisphere SAX column. The eluant was detected using a Canberra Packard A-500 Flo-One on-line *beta* radiodetector where it was mixed in a ratio of 1:3 with Flo-Scint IV scintillation cocktail, according to manufacturer's specifications. The results were analysed on a FLO-one data program. The retention times were compared to standards of [H^3]PI (4) *P* and [H^3]PI (4,5) P_2. The identity of the various peaks obtained using this separation technique has been defined previously (Stephens *et al.*, 1991). The PI (3) *P* elution time was compared to that determined by Dr. Stephen Ward by immunoprecipitating PI 3-kinase using an anti-p85 antibody and performing an *in vitro* lipid kinase assay. The PI (3) P was isolated by TLC and then extracted from the TLC plate and its elution time on the HPLC monitored (personal communication) (Ward *et al.*, 1995). The elution times of the other 3-phosphorylated lipids were compared to those quoted in the literature (Stephens *et al.*, 1991 and Traynor-Kaplan *et al.*, 1988). The levels of PI were used as an internal standard to confirm that each sample contained a similar amount of radioactivity as the PI pool should not vary upon agonist stimulation.

2.2.7.2 Immunoprecipitation and *in vitro* lipid kinase assay

2.2.7.2.1 Coupling the antibody to protein G sepharose beads

The protein G sepharose beads from Sigma were provided in methanol and were therefore washed three times with 1 ml of PBS and then resuspended as a 50% suspension in PBS. 500µl aliquots were stored at 4°C until required. 50µl of anti-p85 antibody was added to 500µl of 50% protein G bead suspension and the volume increased to 1ml with PBS. The suspension was rotated for 2 h at room temperature. Following rotation, the bead suspension was washed three times with 1ml of PBS and again resuspended as a 50% suspension by the addition of 250µl of PBS and stored at 4°C until required.

To confirm that the antibody had coupled to the beads, 10µl of the beads were denatured by heating to 90°C for 10' in reducing sample buffer. The samples were then separated by 10% SDS-PAGE, as described above. The gel was stained with Coomassie blue stain for 6 h and subsequently destained for 24 hours, in order to detect the heavy and light chains of the antibody.

2.2.6.2.2 Immunoprecipitation of the PI 3-kinase.

This was performed as described by (Ward *et al.*, 1991). HT-29 cells were stimulated and lysed as described above. 20µl of lysate was kept for a total kinase sample and the rest of the lysates were precleared with protein G sepharose beads.

The pre-cleared samples were centrifuged at 10 000xg for 5' and the supernatants removed and added to Eppendorf tubes containing 20µl of the antibody-coupled protein G beads. These suspensions were rotated for 2h at 4°C. Thereafter, the immunoprecipitates were

washed 3 times with lysis buffer, once with PBS, twice with 500mM lithium chloride (LiCl) (pH 7.4), once with water and once with lipid kinase buffer, spinning at 10 000xg between each wash. After the final wash, surplus kinase buffer was removed using a Hamilton syringe.

2.2.6.2.3 *In vitro* lipid kinase assay.

Each of the samples, including the total lysate sample, was resuspended in 40µl of lipid kinase buffer. 50µl of the lipid substrate mixture was added to the immunoprecipitates. The reaction was initiated by the addition of 5 µCi of [γ-^{32}P]-ATP (S.A. 3000Ci/mmol, 0.5mCi/ml, 18.5MBq) and 100µM ATP. The samples were incubated in a 25°C water bath for 15' and the reaction quenched using 100µl 1M HCl and 200µl 1:1 chloroform:methanol. The samples were spun for 30" at 10 000xg and then the lower chloroform layer removed and dried *in vacuo*.

The dried samples were resuspended in 50 µl chloroform and applied to a 1% oxalate sprayed thin layer chromatography (TLC) plate. The plate was placed in a TLC tank that had been equilibrated for at least 6 h with propan-1-ol:2N glacial acetic acid (65:35 (v/v)) and lined with filter paper to ensure adequate vapour equilibration. The TLC plate was allowed to run for 15 hours.

Thereafter, the plate was air dried and exposed to iodine to detect the substrates, and finally exposed to a film for 1-12 hours at -70°C. The film was developed using an RGII Fuji X-ray film processor. The total lysate sample contained not only PI 3-kinase but also other kinases such as PI 4-kinase. This provided a positive control for the *in vitro* kinase assay to confirm that the assay was working.

The PtdIns (3) *P* product visualised by autoradiography was confirmed from previous experiments performed by Dr. Stephen Ward, in which the product was extracted from the TLC plate and analysed by HPLC (personal communication).

2.2.8 Detection of Apoptosis

In order to investigate whether HT-29 cells were undergoing programmed cell death and not necrosis, use was made of the relatively late apoptotic event of DNA fragmentation, whereby double stranded DNA is cleaved into mono- and oligonucleosomes which are histone-associated. Two of the methods described below utilise this event.

A relatively early apoptotic event is the externalisation of phosphatidylserine (PS) residues from the inner part of the plasma membrane to the outer surface, in order to facilitate recognition and phagocytosis of the apoptotic cell by neighbouring cells or macrophages and prevent inflammation. Annexin V is a Ca^{2+}-dependent phospholipid-binding protein with high affinity for PS and can be used as a probe for PS exposure and therefore to detect apoptotic cells.

Alternatively, due to the fact that cysteine proteases play an essential role in the onset of apoptotis, detection of protease activity in crude cell lysates from induced cells allows for another method of monitoring and confirming the apoptotic process in HT-29 cells.

2.2.8.1 Apoptag Direct, *In Situ* Apoptosis Detection Kit, Fluorescein

Apoptosis was determined by direct fluorescence detection of end-labelled genomic DNA using the above kit. Residues of fluorescein-nucleotide are catalytically added to the DNA by terminal deoxynucleotidyl transferase (TdT), an enzyme which catalyses a template-independent addition of nucleotide triphosphate to the 3'-OH ends of double- or single-stranded DNA. The incorporated nucleotides form a random heteropolymer, which, when excited by light of 494nm wavelength, the fluorescein generates an intense signal at 523nm. Treated and untreated cells were spun onto slides, fixed with 4% (v/v) neutral buffered formalin, equilibrated and stained with working strength TdT, according to the manufacturer's instructions. As a negative control, some reactions were performed without the TdT enzyme. After washing in stop/wash buffer and PBS, slides were counterstained with propidium iodide staining solution and mounted with glass coverslips. The slides were observed under a fluorescence microscope. The results were scored by counting apoptotic cells (green) and viable cells (red) randomly in various fields. For each condition, 500 to 1000 cells were counted.

2.2.8.2 Cell Death Detection ELISA^{PLUS}

The detection of apoptosis was performed by determining the histone-associated DNA fragments (mono- and oligonucleosomes) generated by the apoptotic cells using the above kit. 10^4 cells/well were aliquoted into 96-well plates and allowed to adhere overnight. Cells were then treated, as indicated, pelleted and the supernatants were removed. Cell pellets were lysed (lysis buffer included in kit) and assayed according to the manufacturer's instructions. Briefly, 20 µl of lysate was transferred into the strepdavidin-coated MTP along with a positive control (provided). 80 µl of the immunoreagent was added to each well, the plate was covered and incubated at room temperature for 2 hours. The plate was shaken

periodically during incubation. The solution was then removed and the plate was washed 3X with incubation buffer. 100 µl of substrate solution was added for 15' before photometric analysis was performed at 405 nm against the substrate solution as a blank (reference wavelength 490 nm), using a microtiter plate-reader (Dynatech MR5000). The specific enrichment of mono- and oligonucleosomes released into the cytoplasm was calculated using the following formula:

$$\frac{\text{mU of the sample (dying/dead cells)}}{\text{mU of control sample (cells without treatment)}} = \text{enrichment factor}$$

(mU = absorbance [10^{-3}])

2.2.8.3 Annexin-V-Fluos

In some experiments, apoptosis was detected by measuring the externalisation of phosphatidyserine by FITC-labeled annexin-V binding using the Apoptosis Detection Kit. Cells were treated as indicated and stained according to the manufacturer's instructions. Briefly, after stimulation cells were dissociated from the wells using Trypsin-EDTA, washed in PBS and centrifuged at 200xg for 5'. The cell pellet was resuspended in 100 µl of labelling solution and incubated at room temperature for 10 - 15' in the dark. After the addition of 400 µl incubation buffer, analysis by flow cytometry (Becton Dickinson FACS Vantage) was performed. Percentage increases in annexin-V- fluoroscein binding above controls were measured.

2.2.8.4 Detection of Caspase Activity

Activation of the caspase cascade is pivotal to the death execution phase of apoptosis and it appears that caspase-8 is the apical member of the apoptotic pathway with caspase-3 lying

downstream (G. M. Cohen, 1997). Colorimetric Protease Assay Kits for both caspase-8 and -3 (Chemicon, USA) allow for the detection of protease activity at two distinct points in the apoptotic cascade. The principle of the assay is based on spectrophotometric detection of the chromophore *p*-nitroanilide (*p*-NA) after cleavage from the labelled tetrapeptide substrate DEVD-*p*NA in the case of caspase-3 and IETD-*p*NA in the case of caspase-8. 10^4 cells/well were aliquoted into 96 well plates and allowed to adhere overnight. Cells were then treated as indicated and pelleted after 24 hours to include any floating cells. The supernatants were removed and the cell pellets were lysed and assayed according to manufacturer's instructions. Briefly, after centrifugation to remove cell debris, 50 µl of cell lysate was transferred into a clean MTP and 50 µl of 2X reaction buffer (provided) containing 10 mM DTT was added. 5 µl of the 4 mM DEVD-*p*NA or IETD-*p*NA substrate (200 µM final concentration) was added and the plate incubated at 37°C for 2 hours. The *p*NA light emission was quantified using a microtiter plate reader at 405nm. Comparison of the absorbance of *p*NA from the apoptotic sample with uninduced control determined the fold increase in caspase activity.

2.2.9 Protein Kinase B Assays

2.2.9.1 Western Analysis

Aliquots of cell lysate supernatant were boiled in 5X sample buffer and electrophoresed through 12.5% (v/v) acrylamide gels (with an acrylamide:bis-acrylamide ratio of 37.5:1) by SDS-PAGE and the proteins were transferred by electroblotting onto nitrocellulose as described above. The blots were probed with a phosphospecific PKB (0.5 µg/ml) which only has affinity for the active, ^{473}Ser-phosphorylated forms of PKB antibody and proteins visualised by ECL with a goat anti-rabbit Ig (0.1µg/ml) conjugated with horseradish

peroxidase as a secondary antibody. Where appropriate, blots were completely stripped of antibodies by incubation at 55°C for 60 minutes with stripping solution. After extensive washing, blots were reblocked prior to reprobing with anti-PKB antibody (0.5 µg/ml).

2.2.9.2 *In vitro* kinase assays

Cells were stimulated as described above and PKBα was immunoprecipitated from cell lysates with 1µg anti-PKBα (Santa Cruz) for 1 hour at 4°C. Immunocomplexes were captured with 30µL of 50% suspension Protein G Sepharose at 4°C with rotation for 1 hour. Beads were washed twice in lysis buffer, twice in LiCl wash buffer and once in kinase buffer. Kinase assays were performed by suspending beads in kinase buffer containing 2.5µg histone H2B, 0.5µM protein kinase inhibitor (PKI), 50µM ATP, 3µCi ATP[γ-^{32}P] at room temperature for 30'. Reactions were stopped by the addition of 5x SDS PAGE sample buffer containing 2-mercaptoethanol followed by boiling for 5'. Samples were electrophoresed in 12.5% polyacrylamide gels as described above. The gel was cut in the middle so that the upper part contained the 60kDa PKBα protein and the lower part the phosphorylated 15 kDa histone H2B protein. The upper part was transferred as described above and immunoblotted for PKBα and the lower part was stained for 15' with Coomassie Blue to detect histone H2B and after destaining, the gel was dried and phosphorylated histone H2B detected by autoradiography at -70°C.

2.2.10 Detection of Bad phosphorylation

Bad was immunoprecipitated from cell lysates with 4 µg of anti-Bad mAb (Transduction Labs). After addition of 30 µl protein G-Sepharose beads (50% suspension in PBS), immunoprecipitates were rotated for 1 h at 4°C and then washed three times in lysis buffer, resuspended in 5X sample buffer and boiled for 5' prior to electrophoresis through 12.5 %

(v/v) acrylamide gels (with an acrylamide:bis-acrylamide ratio of 118:1). The proteins were transferred onto nitrocellulose and Bad was immunoblotted with 0.5 μg/ml anti-Bad polyclonal antibody (Santa Cruz) and proteins visualised by ECL with a goat anti-rabbit Ig (0.1μg/ml) conjugated with horseradish peroxidase as a secondary antibody.

2.2.11 Statistical Analysis

Statistical significance was assessed by two-way analysis of variance followed by Dunnett's test for multiple comparisons with control. Data are expressed as means ± SEM from three independent experiments, unless otherwise stated. $p \leq 0.05$ was taken as the criterion for a significant difference. Throughout **denotes $p \leq 0.01$ and *denotes $p \leq 0.05$.

2.3 BUFFERS AND SOLUTIONS

2.3.1 Solutions and reagents for cell culture

Tissue culture reagents were used for cell cultures and all solutions were prepared using sterile MilliQ water.

Phosphate Buffered Saline, pH 7.4 140 mM NaCl 2.7 mM KCl 1.5 mM KH_2HPO_4 8.1 mM Na_2HPO_4	**Hank's Balanced Salts Solution (HBSS), pH 7.4** 100 ml 10X Hank's balanced salts 1mM Ca^{2+}/Mg^{2+} 0.1% BSA
McCoy's medium 5A Supplemented with penicillin (10 units/ml), streptomycin (10 μg/ml) and fungizone (0.5μg/ml) before use.	**Foetal Bovine Serum** Stored at -20°C in 50 ml aliquots (heat-inactivated).
Freeze medium 10% (v/v) DMSO 40% (v/v) FBS 50% (v/v) McCoy's 5A	

2.3.2 Solutions and buffers for Nitrite assay

HCl solution	**NaOH solution**
0.62 M HCl in Milli-Q water.	2.8 M NaOH in Milli-Q water.

2.3.2 Solutions and buffers for Northern Analysis

DEPC- treated water or buffer Milli-Q water or buffer was treated with 1ml of diethyl pyrocarbonate per litre of water and incubated overnight at 37C. Autoclave (for 20 minutes at 121C).	**20% (w/v) SDS solution** 20g SDS diluted in 100ml sterile Milli-Q water
2M Tris-HCl stock solution, pH 8 2M Trizma base in Milli-Q water, adjust to pH 8 with HCL. Autoclave	**0.5M EDTA stock solution, pH 8** 0.5M EDTA in Milli-Q water, adjust pH. Autoclave
3M Sodium acetate, pH 5.2 3M sodium acetate dissolved in 250 ml Milli-Q water. Adjust pH with 3M glacial acetic acid. DEPC treat and autoclave.	**75% (v/v) ethanol solution** 75 ml absolute ethanol in 25 ml Milli-Q water.
20X MOPS running buffer 0.4M 3-[N-morpholino]-propane-sulfonic acid (MOPS) 0.02M EDTA (4 ml of 0.5M stock/100 ml) 0.2M sodium acetate (6.64 ml of 3M stock/100ml) Adjust volume to 100 ml with Milli-Q water and pH with solid NaOH. Filter sterilise	**RNA sample buffer (per sample)** 7 µl 36% (w/v) formaldehyde 4 µl 20X MOPS running buffer 2 µl 1mg/ml ethidium bromide solution 20 µl formamide Made up fresh before use.
1X MOPS running buffer 50 ml 20X MOPS in 950 ml DEPC-treated water.	**1mg/ml Ethidium bromide solution** 10 mg ethidium bromide in 10 ml DEPC-treated water.
Bromophenol solution 0.025g bromophenol blue 3 ml glycerol Adjust volume to 10 ml with DEPC-treated water	**20X SSC, pH 7** 3M NaCl 0.3M trisodium citrate dihydrate Milli-Q water, DEPC treat and autoclave
Buffer 1 0.1M maleic acid 0.15M NaCl Adjust to pH 7.5 with solid NaOH, DEPC treat and autoclave.	**Blocking stock solution** 10g blocking reagent in 100 ml buffer 1. Microwave to dissolve (do not boil). Autoclave.

Hybridisation solution 5X SSC (25 ml of 20X stock/100 ml) 0.1% sarcosyl (0.33 ml of 30% stock/100 ml) 0.02% SDS (0.1 ml of 20% stock/100 ml) 1% blocking buffer (10 ml of 10% stock/100 ml) in DEPC-treated water.	**2X SSC, 0.1% SDS solution** 50 ml of 20X SSC 2.5 ml of 20% SDS stock Adjust volume to 500 ml with DEPC-treated water.
1X SSC, 0.1% SDS solution 2.5 ml of 20X SSC 2.5 ml of 20% SDS stock Adjust volume to 500 ml with DEPC-treated water.	**Buffer 3, pH 9.5** 0.1M Tris 0.1M NaCl in sterile Milli-Q water.
Washing buffer 0.3% (v/v) Tween-20 in buffer 1.	**Buffer 2** 10% (v/v) blocking stock solution in buffer 1.

2.3.4 Solutions and buffers for SDS-PAGE and Western blotting

Lysis buffer 50 mM Tris pH 7.5 (stock 1M) 150 mM NaCl (stock 5M) 1% (v/v) Nonidet P40 (stock 10% (v/v)) 10% glycerol 5 mM EDTA (stock 0.5M, pH 8.0) 1 mM sodium orthovanadate 1 mM sodium molybdate 10 mM sodium fluoride 40 µg/ml phenylmethylsulfonyl fluoride (PMSF) 1 µg/ml pepstatin A (stock in methanol) 10 µg/ml aprotinin 10 µg/ml leupeptin 10 µg/ml soyabean trypsin inhibitor Milli-Q water	**Stock solutions** 1M Tris-HCl pH 8.8 (60.6 g trizma base/500 ml Milli-Q H_2O) 1M Tris-Hcl pH 6.8 (60.6 g trizma base/500 ml Milli-Q H_2O) Acrylamide/bis acrylamide (30:0.8) (store at 4°C) 10% (w/v) SDS (100 ml, 10 g SDS + 91 ml Milli-Q H_2O) 10% (w/v) ammonium persulphate (1 ml aliquots in Milli-Q H_2O, store at 4°C) TEMED
SDS-PAGE running buffer 25 mM Trizma base 192 mM glycine 0.1% (w/v) SDS Milli-Q water, no need to pH, should be above pH 8.3	**5X SDS-sample buffer** 5% SDS 50% glycerol 200mM Tris-HCl pH 6.8 Milli-Q water Bromophenol blue 5% 2-mercaptoethanol (50µl/ml) or 10% 1M DTT (for reducing buffer)

Semi-dry transfer buffer 39 mM glycine 48 mM Trizma base 0.0375% SDS 20% (v/v) methanol	**Tris-buffered saline (TBS)** 20 mM Tris-HCl pH 7.5 150 mM NaCl Milli-Q water, pH 7.5
Tris-buffered saline (TBSN) TBS + 0.05% (v/v) NP40	**Ponceau S** 0.1% (w/v) Ponceau S 5% (v/v) acetic acid
Blocking buffer (a) For most antibodies: 5% (w/v) non-fat powdered milk (Marvel) in TBS 0.05% azide (b) For anti-phosphotyrosine immunoblots: 5% BSA (w/v) 1% ovalbumin (w/v) 0.05% azide	**Coomassie blue stain** 0.25% (w/v) Coomassie blue 45.4% (v/v) methanol 9.2% (v/v) glacial acetic acid Milli-Q water
Stripping buffer 62.5 mM Tris-HCl, pH 6.8 2% (w/v) SDS 100 mM 2-mercaptoethanol Milli-Q water	**Destain solution** 45.4% (v/v) methanol 9.2% (v/v) glacial acetic acid Milli-Q water

Recipes for various percentage gels

Resolving gel – 5 ml is sufficient for 1 mini gel and 40 ml for 1 large gel

Stacking gel* – 1.5 ml is sufficient for 1 mini gel and 8 ml for 1 large gel

Final % gel	5%*	7.5%	10%	12%	15%
Range (kDa)		70-200	20-100	10-70	8-50
Acrylamide	1.67	3.75	5	6	7.5
Milli-Q H_2O	6	5.6	4.35	3.35	1.85
1M Tris pH 8.8		5.6	5.6	5.6	5.6
1M Tris pH 6.8	1.25				
10 % SDS	0.15	0.25	0.25	0.25	0.25
Total (ml)	9.07	15.2	15.2	15.2	15.2

Plus 50µl 10% APS

Plus 20µl TEMED

Antibodies used for immunoprecipitation and immunoblotting

Assay	Primary antibody	Concentration	Secondary antibody	Protein beads
In vitro lipid kinase Western blotting	p85α mAb	1:10 1 µg/ml	anti-mouse HRP	A-sepharose
Western blotting	p85 (SH3/bcr)	0.5 µg/ml	anti-rabbit- HRP	
Immunoprecipitation Western blotting	IRS-1 pAb	1 µl/ml 0.5 µg/ml	anti-rabbit- HRP	A-sepharose
Immunoprecipitation Western blotting	IRS-2 pAb	1 µl/ml 0.5 µg/ml	anti-rabbit- HRP	A-sepharose
Antiphosphotyrosine Western blotting	4G10 mAb	0.5 µg/ml	anti- mouse-HRP	
In vitro kinase	PKB-α pAb	1µg/ml	anti- rabbit-HRP	G-sepharose
Western blotting	PKB-α pAb	0.5 µg/ml	anti- rabbit-HRP	
Western blotting	PKB-α ^{473}Ser	0.5 µg/ml	anti- rabbit-HRP	
Western blotting	PKB-α pAb	0.5 µg/ml	anti- rabbit-HRP	
Immunoprecipitation	Bad mAb	4µg/ml		G-sepharose
Western blotting	Bad pAb	0.5 µg/ml	anti- rabbit-HRP	
Western blotting	Bak pAb	0.5 µg/ml	anti- rabbit-HRP	
Western blotting	p70S6K pAb	0.5 µg/ml	anti- rabbit-HRP	
Western blotting	p70S6K ^{411}Ser	0.5 µg/ml	anti- rabbit-HRP	
Western blotting	p70S6K ^{421}Thr/^{424}Ser	0.5 µg/ml	anti- rabbit-HRP	
Immunoprecipitation	SHIP/SH2- GST	10 µg/ml		Glutathione- sepharose
Western blotting	Shc pAb	0.5 µg/ml	anti- rabbit-HRP	
Western blotting	SHPTP2 pAb	0.5 µg/ml	anti- rabbit-HRP	

2.3.5 Solutions and buffers for PI 3-kinase assays

Phosphate buffer 1.25M *di*-ammoniumhydrogen orthophosphate, $(NH_4)_2HPO_4$ Milli-Q water pH adjusted to 3.8 using orthophosphoric acid	**Lithium chloride** 500 mM LiCl, pH 7.4
***In vitro* lipid kinase buffer** 5 mM $MgCl_2$ 0.25 mM EDTA 20 mM Hepes pH adjusted to 7.4	**Lipid substrate mixture** 1 mg of phosphatidylinositol 1 mg of phosphatidylserine Made up in 2ml of 25mM Hepes/1mM EDTA Dispersed by sonication 3X 15 second bursts on ice)

2.3.6 Solutions and buffers for apoptosis assays

Phosphate buffered saline 50 mM sodium phosphate, pH 7.4 200 mM NaCl	**Stop/wash buffer** 1 ml Stop/wash buffer, provided in kit 34 ml Milli-Ro water 35 ml Total Store at 4°C.
Working strength TdT Amount for 5 cm^2 (ie., per slide) 38 µl Reaction buffer, provided in kit 16 µl TdT enzyme 54 µl Total Vortex and keep on ice.	**Propidium Iodide staining solution** PI stock solution of 50 µg/ml. For 10 mls: propidium iodide (50 µg) RNase A (50 U) (0.5 mg) PBS, pH7.4 (10 ml) Make up fresh and keep on ice.
Immunoreagent For 10 tests: anti-histone-biotin mAb 40 µl anti-DNA-peroxidase mAb 40 µl incubation buffer, provided 720 µl Total 800 µl Vortex and use immediately.	**Incubation buffer** 10 mM Hepes/NaOH, pH 7.4 140 mM NaCl 5 mM $CaCl_2$
Substrate solution ABTS substrate tablet, (2,2'-azino-di-(3-ethylbenzathiazoline sulfonate) 15 ml sustrate buffer, provided in kit Protect from light.	**Annexin-V-Fluos labelling solution** Annexin-V-Fluos labelling reagent 20 µl Incubation buffer 1 ml PI (from stock) 20 µl

2.3.7 Solutions and buffers for PKB assays

LiCl wash buffer	**Kinase buffer**
500mM LiCl	50 mM Tris-HCl pH 7.5
100mM Tris HCl pH 7.5	10 mM $MgCl_2$
1mM EDTA	1 mM DTT

3. MODULATION OF INDUCIBLE NITRIC OXIDE SYNTHASE EXPRESSION AND ACTIVITY

3.1 RESULTS

Cytokine-induced iNOS activity

During the pathogenesis of IBD, human colonic epithelial cells express inflammatory mediators, such as chemokines and nitric oxide. Increased pro-inflammatory cytokines circulate in the underlying gut mucosa and exert their effects on many cell types, including epithelial cells. Using HT-29 cells as model colon epithelial cells, initial investigations sought to confirm the mechanisms involved in nitric oxide production and regulation by T cell-derived cytokines as proposed by Kolios *et al*, 1995.

Growth-arrested monolayers of HT-29 cells, when stimulated with vehicle produced a small constitutive amount of nitrite (Fig. 3.1A). The pro-inflammatory cytokines IL-1α (10 ng/ml), TNF-α (100 ng/ml) and IFN-γ (300 U/ml) added alone to HT-29 cells did not induce a significant increase in nitrite generation after 24h as compared to the constitutive nitrite production in vehicle treated cells (Fig. 3.1A). The combination IL-1α/IFN-γ was the minimal requirement for enhanced nitrite production, whilst other pairs of cytokines were ineffective. Stimulation with IL-1α/IFN-γ produced a significant ($p < 0.01$) increase in nitrite production of 175 ± 24 nM/10^6 cells (n=3) at 24 hours, compared to the basal production of 45 ± 8 nM/10^6 cells (n=3) (Fig. 3.1A). The addition of TNF-α to the combination of IL-1α/IFN-γ produced approximately a 3 fold enhancement of IL-1α/IFN-γ induced nitrite generation, i.e., 384 ± 35 nM/10^6 cells (n=3) (Fig. 3.1A).

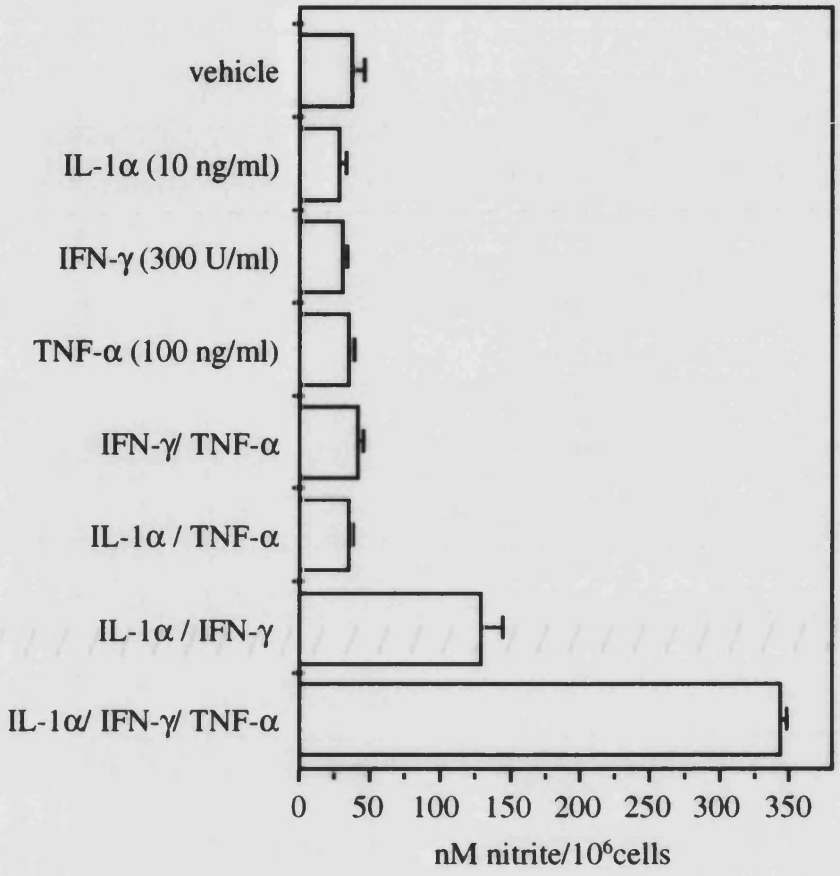


Figure 3.1A. Growth-arrested HT-29 monolayers were stimulated with cytokines, as indicated. After 24 hours, nitrite in the supernatants was measured using the fluorometric nitrite assay, see section 2.1.1. Each point represents the mean ± SEM of at least three separate experiments.

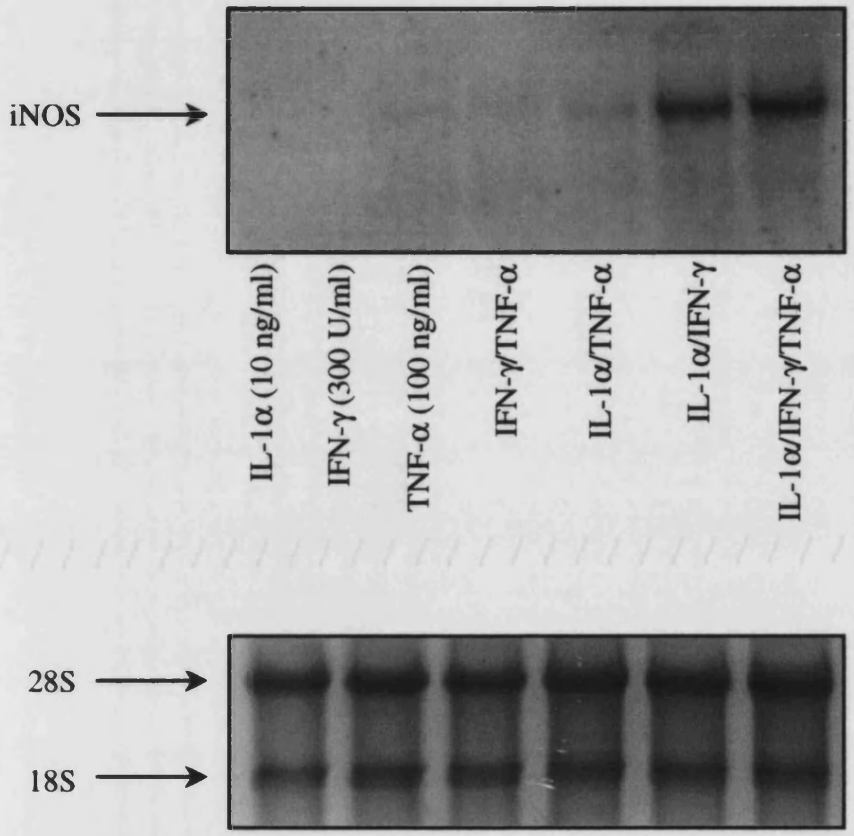


Figure 3.1B. Growth-arrested HT-29 monolayers were stimulated with cytokines, as indicated. After 24 hours, cells were harvested for Northern analysis, as described in Section 2.3. The top panel shows the iNOS mRNA detected and the bottom panel indicates equal loading of total RNA. Data is representative of three experiments.

To determine whether the inducible generation of nitric oxide by HT-29 cells was due to the induction of iNOS gene transcription, the iNOS mRNA was determined by Northern analysis after 24 hours stimulation. In cells stimulated with vehicle or individual pro-inflammatory cytokines, iNOS transcripts were not detected (Fig. 3.1B). Similar to the nitrite measurements, the combination IL-1α/IFN-γ was the minimal requirement for iNOS expression, whilst other pairs of cytokines were ineffective. However, the addition of TNF-α to the combination of IL-1α/IFN-γ was without effect on the IL-1α/IFN-γ-induced iNOS mRNA expression in these cells (Fig. 3.1B), suggesting that the up-regulation by TNF-α on the IL-1α/IFN-γ-induced nitrite generation by HT-29 cells is at the post-transcriptional level.

Regulation of iNOS activity by NOS inhibitors

The potential therapeutic role for iNOS inhibitors has led to their use in animal experimental models. Non-selective agents able to inhibit both ecNOS and iNOS seem to be effective in some experimental models (Hogaboam *et al.*, 1995; Rachmilewitz *et al.*, 1995 and Seago *et al.*, 1995). However, suppression of ecNOS achieved by these agents can actually worsen intestinal injury (Pfeiffer and Qiu, 1995 and Dobosz *et al.*, 1996) and prevent epithelial repair (Miller *et al.*, 1993). However, selective inhibition of iNOS in a model of chronic colitis in monkeys did not reduce either histological inflammation or diarrhoea (Ribbons *et al.*, 1997). It is possible that iNOS inhibitors are effective as protective agents only when administered at the time of induction of intestinal inflammation and do not exert substantial anti-inflammatory activities when administered after inflammation has already been established and iNOS expressed (Ribbons *et al.*, 1997). Thus, the effect of various iNOS inhibitors on cytokine-induced iNOS activity was measured by treating HT-29 cells with

iNOS-inducing combinations of pro-inflammatory cytokines concomitantly with available iNOS protein inhibitors. This different approach was thought could verify that the activity of iNOS was contributing to the nitrite being measured in the culture supernatant.

Co-stimulation of HT-29 cells with both pro-inflammatory cytokines and iNOS inhibitor resulted in a 75 ± 3 % (n=3) inhibition of nitrite accumulation by AG and a 91 ± 2 % (n=3) inhibition by NMMA (Table 3.1). The difference may be accounted for if one considers that NMMA may be concurrently inhibiting ecNOS. 1400W, at the higher concentration of 100μM produced a 75 ± 4 % (n=3) inhibition of nitrite accumulation (Table 3.1), with the lower concentration of 10 μM only just producing a significant inhibition.

Induction of remission is the first line of defence in the management of IBD symptoms and the therapeutic use of anti-inflammatory agents is widespread (W. J. Sandborn, 1998 and D'Haens and Rutgeerts, 1998). To investigate whether these agents play a role in iNOS activity, two types of agent in common use were added to HT-29 cells 30 minutes prior to cytokine induction of iNOS.

Dexamethasone, a corticosteroid, is an anti-inflammatory drug that acts transcriptionally by binding to a cytosolic glucocorticoid receptor and then translocating to the nucleus and interacting directly in the promoter region of target genes. It is a known iNOS inhibitor in a wide variety of cell types and is thought to have both transcriptional and posttranscriptional effects (Radomski *et al.*, 1990, Rachmilewitz *et al.*, 1995 and Rees *et al.*, 1995). However, in the HT-29 system, dexamethasone (1 – 10 μM) had no effect on nitrite accumulation over 24 hours post induction (Table 3.2).

5-Aminosalicylate (5-ASA) is a nonsteroidal anti-inflammatory drug (NSAID) widely used for patients with active IBD. It has been shown to inhibit the activity of iNOS (J. F. Valentine, 1998 and Reynolds *et al.*, 1995), although NO generation in cultured tissues from colitic patients was unaffected (Rachmilewitz *et al.*, 1995). Interestingly, 250 μM of 5-ASA given prior to the addition of the iNOS-inducing cocktail of cytokines, gave no significant change in the nitrite accumulated over 24 hours (Table 3.2).

Sodium salicylate (4mM) is also an NSAID shown to inhibit transcription of iNOS and subsequent nitrite accumulation (Farivar *et al.*, 1996). Interestingly, although a significant decrease in nitrite accumulation was seen (Table 3.2), more than 50 % of the HT-29 cells were found detached after 24 hours, perhaps suggesting that the concentration may be cytotoxic or this drug has an apoptotic mode of action. Also, it should be noted that the concentration may not reflect the physiologically relevant range. The concentration for sodium salicylate was taken from Farivar and Brecher, 1996.

Table 3.1. Confluent HT-29 cells were starved of serum for 24 hours prior to treatment with IL-1α/IFN-γ/TNF-α, with concurrent addition of the iNOS inhibitor, as listed. Supernatants were collected after 24 hours and assayed for nitrite as described in Section 2.2.3. * denotes $p<0.001$

NOS inhibitor	% inhibition in nitrite
Aminoguanidine (AG) (500 μM)	75* ± 3 %
NMMA (500 μM)	91* ± 2 %
1400W (10 μM)	20 ± 5 %
1400W (100 μM)	75* ± 4 %

Table 3.2. Confluent HT-29 cells were starved of serum for 24 hours prior to a 30 minute incubation with the therapeutic agent as listed, followed by stimulation with IL-1α/IFN-γ/TNF-α. Supernatants were collected after 24 hours and assayed for nitrite as described in Section 2.2.3. * denotes $p<0.001$

Therapeutic agent	% inhibition in nitrite
Dexamethasone (1 – 10 μM)	no significant change
5-ASA (250 μM)	no significant change
Sodium salicylate (4mM)	88* ± 3 %

The regulation of iNOS activity by other pharmacological agents

Existing data relating the role of iNOS to gut dysfunction have been obtained primarily in rodent models (Salzman, 1995) and may generalise imperfectly to the clinical setting because of differences in iNOS transcriptional regulation, cofactor requirements and susceptibility to pharmacological inhibitors. Given the importance of preserving gut barrier function in critical illness, agents that modulate the induction or activity of iNOS in the intestinal epithelium may have therapeutic potential.

Phorbol 12-myristate 13-acetate (PMA) mimics diacylglycerol (DAG) and is, thus, a direct activator of PKC, particularly the typical and novel isoforms. The role of kinases in the transcription of iNOS is not completely understood and this investigation sought to ascertain whether PKC activation could lead to changes in nitrite accumulation, which might indicate a change in iNOS expression. As shown in Table 3.3, both doses of 5 ng/ml and 50 ng/ml PMA caused a highly significant enhancement of nitrite present in the supernatants. Interestingly, the higher dose of 50 ng/ml resulted in an approximate 20 % reduced enhancement from that of the 5 ng/ml dose, perhaps suggesting that more chronic treatment by this phorbol ester might lead to the inhibition of certain PKC isoforms.

The MAPK pathways are involved in a number of cell signalling pathways. The availability of SB203580, a specific inhibitor of p38, has made it possible to identify effects mediated by or requiring active p38 protein kinase (Cuenda *et al.*, 1995). Similarly, the availability of another inhibitor, PD098059, which inhibits MEK1 and MEK2 activation, has been used to study ERK-mediated responses (Dudley *et al.*, 1995). Indeed, the use of these inhibitors was used in this study to investigate a role for these kinases in iNOS activity. SB203580 at

30 μM completely abolished the cytokine-induced nitrite accumulation, whereas PD098059 at 50 μM produced a significant but partial inhibition. These results strongly suggest a requirement for the stress-response pathway in either the expression of iNOS or its activity, although a role for the proliferation/differentiation pathway is also implicated.

Another important intracellular signalling pathway, already discussed in Section 1.5, is the PI 3-kinase pathway activated by growth and survival factors. At the time of this investigation there was no evidence to suggest that this family of lipid kinases had a role to play in NOS expression or activity. However, the Class I_A of PI 3-kinases also has protein serine kinase activity and the physiological role for this function has yet to be investigated. Hence, the fungal metabolite wortmannin, which irreversibly inhibits both the lipid and serine kinase activity of PI 3-kinase at 100 nM, and the structurally unrelated compound LY294002, which may be more specific, were used to investigate the role of PI 3-kinase on iNOS activity. Interestingly, neither inhibitor was able to upregulate iNOS on its own, but there was a differential effect when added with the pro-inflammatory cytokines (Table 3.3). Wortmannin at 100 nM made no significant difference to the cytokine-induced effect on nitrite accumulation, although the higher dose of 300 nM significantly enhanced this effect. Also, LY294002 made no significant difference to this measure of iNOS activity. These data are conflicting, but may indicate the participation of PI 3-kinase at some level of iNOS regulation.

Table 3.3. Confluent HT-29 monolayers were starved for 24 hours, pre-treated with an agent, as listed, for the times indicated and followed by stimulation with the cocktail of cytokines IL-1α/IFN-γ/TNF-α. Supernatants were collected after 24 hours incubation and assayed for nitrite accumulation, as described in Section 2.2.3. * denotes $p<0.01$

Pharmacological agent	**Incubation time (mins)**	**% change in nitrite**
Phorbol Myristate Acetate (PMA) (5 ng/ml)	30	63* ± 5 % enhanced
PMA (50 ng/ml)	30	43* ± 1 % enhanced
SB203580 (3 μM)	60	59* ± 1 % inhibition
SB203580 (30 μM)	60	99* ± 1 % inhibition
PD098059 (50 μM)	60	47* ± 3 % inhibition
Wortmannin (100 nM)	10	21 ± 2 % enhanced
Wortmannin (300 nM)	10	2* ± 2 % enhanced
LY294002 (10 μM)	15	No significant change
LY294002 (30 μM)	15	No significant change

Differential expression and activity of iNOS in the presence of cycloheximide

Cycloheximide is a translation inhibitor, which directly results in the interference of protein synthesis (Obrig *et al.*, 1971). To determine whether *de novo* protein synthesis is a feature of iNOS expression and activity, experiments were performed whereby HT-29 cells were treated with a range of concentrations of cycloheximide one hour prior to the cytokine mix. Lower doses (0.25 - 1 μg/ml) significantly enhanced both nitrite production (Fig. 3.2A) and iNOS mRNA transcripts (Fig. 3.2B). Higher doses (2.5 - 10 μg/ml) inhibited both nitrite production (Fig. 3.2A) and iNOS mRNA (Fig. 3.2B). This is an intriguing result, but it would appear that cycloheximide may have a broader range of targets than originally thought and that different doses may affect different sets of proteins.

Cytokine-induced inhibition of iNOS activity

Pro-inflammatory cytokines are clearly involved in the up-regulation of iNOS and it would be reasonable to assume that anti-inflammatory cytokines may play a role in the down-regulation of either iNOS expression or activity or both. Preliminary data in our laboratory by Dr. George Kolios suggested that the anti-inflammatory cytokines IL-13 and IL-4 had largely inhibitory effects on IL-1α/IFN-γ/TNF-α–induced nitrite generation. In order to confirm this assertion, confluent monolayers of HT-29 cells were pre-treated for 1 hour with increasing doses of IL-13 (0.3 - 30 ng/ml), followed by the combination of cytokines IL-1α/IFN-γ/TNF-α for 24 hours. IL-13 produced a partial, but significant suppression of nitrite accumulation ($p<0.01$- $p<0.001$), (Fig. 3.3), to the levels normally generated by the minimal combination of IL-1α/IFN-γ. This possibly suggests that IL-13 down-regulates the post-transcriptional effect of TNF-α on the IL-1α/IFN-γ–induced nitrite generation.

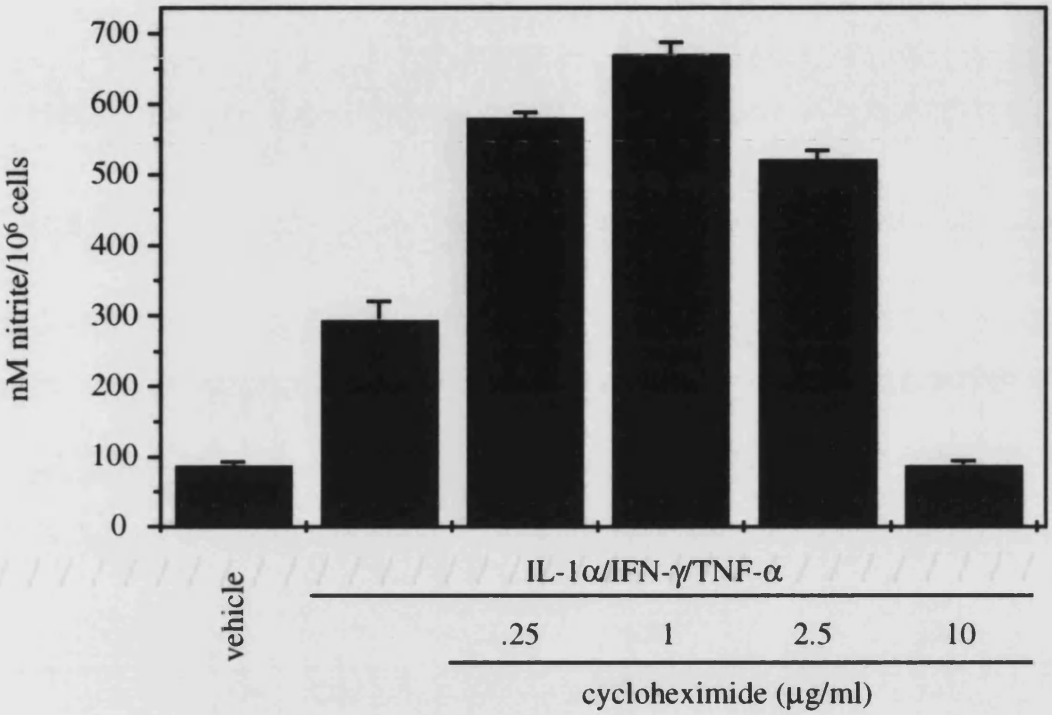


Figure 3.2A. Growth-arrested HT-29 monolayers were stimulated with cycloheximide (μg/ml) for 1 hour prior to the addition of cytokines, as indicated. After 24h, nitrite in the supernatants was measured using the fluorometric nitrite assay. Data is the mean ± SEM, n = 3.

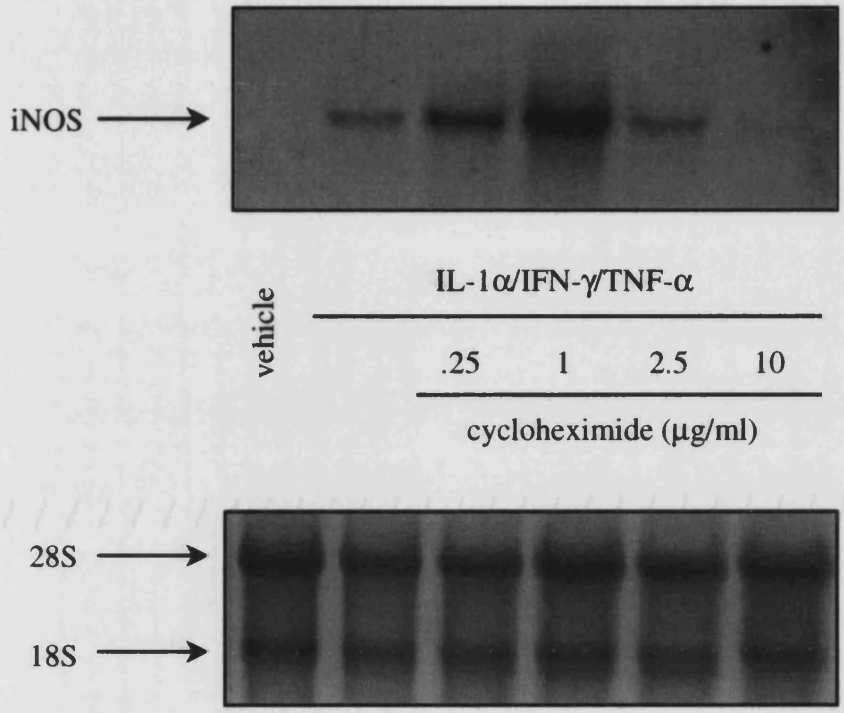


Figure 3.2B. Growth-arrested HT-29 monolayers were stimulated with cycloheximide (μg/ml) for 1 hour prior to the addition of cytokines, as indicated. After 24h, cell were harvested for Northern analysis. The top panel shows the iNOS mRNA detected and the bottom panel indicates equal loading. Data is representative of three experiments.

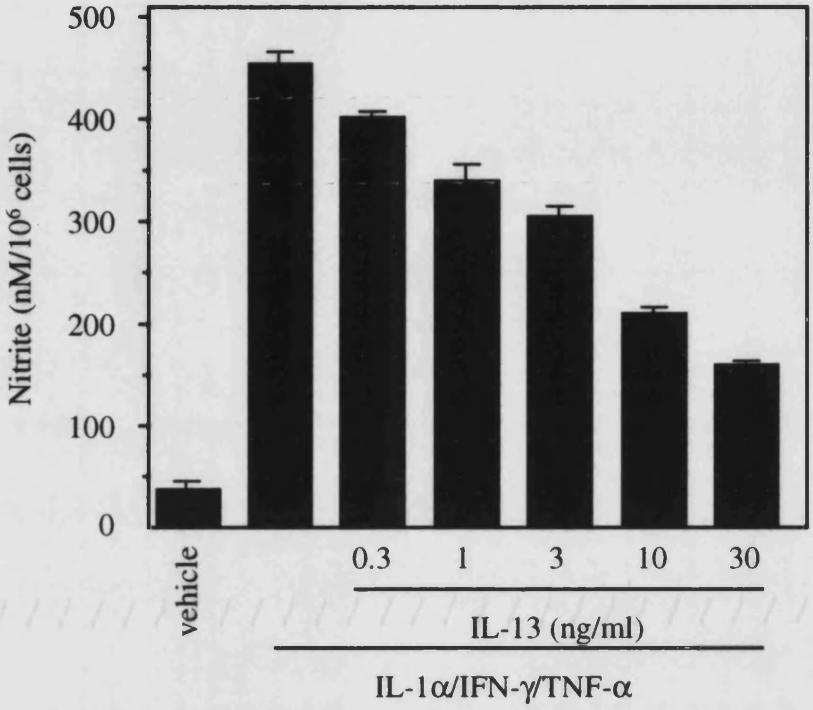


Figure 3.3. Growth-arrested HT-29 cells were stimulated with increasing concentrations of IL-13 for 1 hour prior to the addition of cytokines, as indicated. After 24 hours, nitrite production was measured in the supernatants using the fluorometric nitrite assay. Each point represents the mean ± SEM of three separate experiments

However, it is interesting to note that only the higher concentrations of IL-13 (i.e., 10 - 30 ng/ml) are able to block iNOS mRNA induced by pro-inflammatory cytokines (Dr. George Kolios, personal communication), although the mechanism of this is unclear.

3.2 DISCUSSION

NO is produced at many sites of the gastrointestinal tract and it is considered as an important mediator in physiological and pathological events (M. Guslandi, 1994). At the beginning of this study, NO synthesis (Middleton *et al.*, 1993) and NOS activity had been reported increased in the inflamed mucosa from patients with IBD compared to the uninflamed controls, (Rachmilewitz *et al.*, 1995), but the type of cells responsible for this production was unknown. In our laboratories the source of NO generation had been examined in colonic mucosa using immunohistochemical analysis of human large bowel biopsies, cultures of the colonic adenocarcinoma cell line HT-29 and cultures of human colonic biopsies (Kolios *et al.*, 1995). Immunohistochemical studies clearly showed that iNOS expression was localised to the surface epithelium and crypts and not detectable in the lamina propria or in the inflammatory leukocytes closely associated with the epithelial cells in the mucosa from patients with ulcerative colitis (Kolios *et al.*, 1998). However, biopsies from newly diagnosed ulcerative colitis showed iNOS staining in the apical region of the superficial region of crypts in close association to areas of intense neutrophil infiltration of lamina propria and epithelium (Kolios *et al.*, 1998). Thus, the epithelium appears to be the major source of NO in IBD, but iNOS expression by colonic epithelial cells is only present when there is an underlying inflammatory component within the colonic mucosa.

In support of these findings, it was demonstrated that human HT-29 colonic epithelial cells, in response to specific combinations of cytokines, express iNOS mRNA and produce large quantities of nitrite (Kolios *et al.*, 1995). The expression of iNOS activity in cells and tissues is controlled by a combination of pro-inflammatory cytokines and the profile of cytokine responsiveness varies with cell type. In this study, unstimulated HT-29 cells produced a small amount of nitrite, which may be due to ecNOS as no iNOS was present. None of the cytokines added alone increased the nitrite generation. The combination of IL-1α and IFN-γ was the minimal stimulation required for iNOS mRNA or significant increase of nitrite generation in HT-29 cells, whilst no other pair of cytokines was effective. The combination of IL-1α/IFN-γ/TNF-α was the most effective for increase in nitrite generation, with the effect of the TNF-α signal being at the post-transcriptional level.

It is most likely that the increased nitrite accumulation is due to iNOS enzyme activity because this activity was, 1) induced by a combination of pro-inflammatory cytokines that induce the expression of iNOS mRNA and, 2) blocked by pharmacological iNOS inhibitors, aminoguanidine and L-NMMA and, more specifically, by 1400W. These data suggest that human colonic cells can be considered as an important source of nitric oxide production.

As mentioned earlier, patients with active IBD have an increased secretion of pro-inflammatory cytokines in the mucosa, which may result in the greatly enhanced iNOS activity seen in these patients. The fact that anti-inflammatory drugs routinely used successfully on patients with active disease have variable effects on iNOS activity in HT-29 cells reveals the complex aetiology of IBD. The steroid hormone dexamethasone had no effect on the nitrite produced by epithelial cells. This correlates with a study undertaken by Salzman *et al*, 1996, in which dexamethasone had no effect on cytokine-induced nitrite

accumulation, but they did find a slight, but significant decrease in iNOS mRNA. It has been proposed that dexamethasone decreases transcriptional rate and increases degradation of iNOS mRNA (Kunz *et al.*, 1996) or reduces translation and increases degradation of iNOS protein (Kunz *et al.*, 1996). Transcription factor binding sites in the iNOS promoter region include NF-κB and the activation of this family of proteins is essential for cytokine-induced iNOS transcription (Xie *et al.*, 1994). Glucocorticoids are thought to mediate their effects by inhibition of NF-κB activity, through the induction of IκB synthesis (Scheinman *et al.*, 1995 and Auphan *et al*, 1995). Alternatively, Salzman *et al*, 1996 found that dexamethasone caused a slight, but significant reduction in mitochondrial respiration, although the significance of this was not discussed. Other groups have failed to observe any difference in iNOS expression (Kimura *et al.*, 1998 and Godkin *et al.*, 1996), NO generation (Lundberg *et al.*, 1994 and Boughton-Smith *et al.*, 1993) and peroxynitrite formation (Kimura *et al.*, 1998) between steroid-treated and -untreated patients with UC. The therapeutic activity of dexamethasone might lie in its ability to inhibit iNOS expression and activity only if given at the very beginning of the inflammatory process, their effect being diminished once the inflammation is well under way (Godkin *et al.*, 1996). Alternatively, dexamethasone might mediate its effects through its action on iNOS in leukocytes and not epithelial cells (J. Linehan, personal comm.).

Sodium salicylate has been shown to be a transcriptional inhibitor of iNOS in cultured cardiac fibroblasts (Farivar and Brecher, 1996), although the mechanism is not clearly understood. In HT-29 cells sodium salicylate was cytotoxic and this may account for the reduced levels of nitrite accumulated in the supernatants. Interestingly, sodium salicylate exacerbates IBD and it has been proposed that increased apoptosis is the cause (Elder *et al.*, 1997). Inhibition of the activity of cyclooxygenase, a key enzyme in biosynthesis of pro-

inflammatory prostaglandins (J. R. Vane, 1971), through the inhibition of NF-κB and AP-1 by salicylate (Xu *et al.*, 1999 and Cronstein *et al.*, 1999) could explain the anti-inflammatory effects. How COX2 might play a role in apoptosis is unknown at present, but could explain the result seen in this study. 5-ASA, on the other hand, had no effect on nitrite measurements, although a recent finding suggests that it inhibits the impaired epithelial barrier function induced by IFN-γ (Di Paolo *et al.*, 1996). Additionally, it is thought that 5-ASA acts as a scavenger of reactive oxygen and nitrogen derived species (Miyachi *et al.*, 1987), but a recent study showed that 5-ASA was able to scavenge the potent neutrophil oxidant HOCl and not H_2O_2 or NO (McKenzie *et al.*, 1999). Thus, it would seem unlikely that modulation of colonic NO production is the major mechanism for explaining the therapeutic activity of the drugs currently used in treatment of IBDs.

Enhanced cytokine-induced nitrite generation by PMA suggests a dependency on the PKC pathway. However, there are 11 isoforms of PKC, some of which are sensitive to phorbol esters (namely, the classical and novel subfamilies). The identification of PMA-responsive, non-PKC proteins (e.g., protein kinase D and GTPase-activating proteins, GAPs) raises the need for caution in the interpretation of studies of PKC function that rely solely on the use of phorbol esters as an investigative tool (Mellor and Parker, 1998).

The classical MAP kinase cascade (i.e., Raf/MEK/ERK) can be triggered by phorbol esters and the evidence indicates the involvement of PKC being both necessary and sufficient (Alessandrini *et al.*, 1992). This suggests that PKC activation in the HT-29 system might amplify the signal generated by both IL-1 and TNF-α through this kinase pathway. Both IL-1 and TNF-α activate of the JNK/SAPK and p38 stress response pathways (O'Neill and Greene, 1998 and Sluss *et al.*, 1994), as well as the ERK pathway. This points to an

important role for these pathways in the expression of iNOS. The inhibition of p38 by SB203580 resulted in the complete abrogation of iNOS activity, whilst the MEK inhibitor PD098059 produced only a partial effect. The p38 kinase and its upstream kinases are implicated in responses to cellular stress, inflammation and apoptosis, probably as a result of the activation of transcription factors, such as NF-κB (DaSilva *et al.*, 1997 and Zhao and Lee, 1999). It is possible that the effects of protein kinases may be on the phosphorylation status of iNOS protein, an important feature of iNOS activity. However, Kleinert *et al.*, 1998 showed that only protein tyrosine kinases (in particular, JAK2) were important in the regulation of iNOS in human DLD-1 cells and not serine/threonine kinases.

The use of PI 3-kinase inhibitors (and, indeed, any pharmacological inhibitors or activators) has its limitations due to the broader range of targets that these compounds have. Wortmannin, for example, inhibits PI 3-kinases enzymic function at low nanomolar concentrations (e.g., <10 nM) (Arcaro and Wymann, 1993), but further targets have been identified. It can inhibit phospholipase A_2 (with an IC_{50} of 2 nM) (Cross *et al*, 1995) and mammalian soluble PI 4-kinase (with an IC_{50} of 3-5 nM) (Nakanishi *et al*, 1995). At more elevated concentrations phospholipase D (Reinhold *et al*, 1990) and phospholipase C (Bonser *et al*, 1991) are inhibited. Additionally, wortmannin has been reported to inhibit the mammalian target of rapamycin (mTOR) which regulates p70S6 kinase, a potential PI 3-kinase target (see Section 1.6) (Brunn *et al*, 1996). Also, it is impossible to distinguish which class of PI 3-kinase is relevant because wortmannin inhibits different classes to different extents. Hence, the interpretation that there might be a role for PI 3-kinase in the slight enhancement of iNOS activity induced by the three cytokines has to be tempered with the knowledge that other signalling molecules could be involved, although it may indicate a level of regulation by this enzyme. The novel PI 3-kinase inhibitor, LY294002, appears not

to have significant effects on the activity of other enzymes at 1.4μM, including PI 4-kinase, src, MAP kinase, S6 kinase, DAG kinase, PKA and PKC (Vlahos *et al*, 1994). Taken together, this suggests that there might be a role for PI 3-kinase in the regulation of iNOS activity, although this is unlikely. Certainly, by itself it does not significantly affect the activity of iNOS, but there is a chance that PI 3-kinase inhibition with a simultaneous pro-inflammatory onslaught, may lead to changes in either iNOS activity or transcription. This hypothesis will be addressed in the next section.

By itself, the protein synthesis inhibitor, cycloheximide, did not affect iNOS production at 10 μg/ml. It reduced the nitrite production to constitutive levels, regardless of maximal stimulation with IL-1α/IFN-γ/TNF-α, suggesting that the nitrite evoked by these cytokines is dependent on *de novo* protein synthesis, probably the iNOS enzyme and/or essential peptide cofactors. However, a differential dose response between low and high concentrations was seen in HT-29 cells.

Lower doses significantly enhanced both nitrite production and iNOS mRNA transcripts, perhaps by regulating the phosphorylation state of transcription factors or the activity of cNOS or eNOS. Higher doses inhibited both nitrite production and iNOS mRNA, again indicating the possibility that cycloheximide interferes with the phosphorylation status of proteins already synthesised in the cell. For example, interference with the function of RNAses would affect mRNA stability and/or accumulation. Indeed, it has been shown that a specific sequence in iNOS mRNA mediates its instability and the degradation is prevented by protein-synthesis inhibition (Evans *et al.*, 1994). This would lead to an accumulation of translatable mRNA. Also, the negative feedback loop provided by the iNOS protein would not be present, leading to the accumulation of iNOS mRNA. Oguchi et al, 1994 suggest

that cycloheximide increases the synthesis of iNOS mRNA in macrophages by stimulating the transcription factors c-fos, c-jun and JE. Translation inhibitors such as cycloheximide also activate the p38 kinase of the MAP kinase pathway with resultant stimulatory effects on transcription factor activity. Cycloheximide leads to superinduction of immediate-early genes by the activation of intracellular signalling pathways involving both the MAPK and SAPK cascades. (Zinck *et al.*, 1995). The likely involvement of NF-κB response elements on both basal and inducible transcription of the iNOS gene, would suggest that cycloheximide activation of these components leads to more efficient transcription of the iNOS gene. Casado *et al.*, 1997 showed that cycloheximide potentiates NF-κB activity by increasing degradation of the IκB inhibitory subunit. Alternatively, incompletely blocked protein synthesis may allow translation of mRNAs encoding newly induced transcription factor(s), so that iNOS mRNA accumulation is a secondary, rather than a primary response to cycloheximide-regulated signals in the nucleus.

Since both T-cells and T-cell-derived cytokines, including IFN-γ, IL-4/IL-13 and IL-10, have been detected in the mucosa of patients with IBD (R. B. Sartor, 1994), we have utilised human colonic epithelium (in the form of the HT-29 epithelial cell line) as a model to explore the regulation by IL-13 of epithelial iNOS activity. IL-13 produces a concentration related inhibition of nitrite accumulation induced by the optimal cytokine combination IL-1α/IFN-γ/TNF-α. This inhibition is significant at the high dose of IL-13 (30 ng/ml) and, although only partial, it could be that the result reflects an inhibition of the post-transcriptional effect of TNF-α. It would be prudent to confirm that IL-13 can indeed inhibit iNOS transcription and to investigate the mechanisms of this function. These issues will be addressed in the next section.

In summary, HT-29 cells can be induced by a combination of pro-inflammatory cytokines to express iNOS. Kinase activators and inhibitors, corticosteroids, non-steroidal anti-inflammatory drugs and the anti-inflammatory cytokine, IL-13 can modulate the activity of iNOS. In conclusion, these data highlight the complexity of iNOS expression and activity (see Figure 3.4) and suggest a crucial role for pro-inflammatory and T-cell-derived cytokines in colonic mucosa during intestinal inflammation.

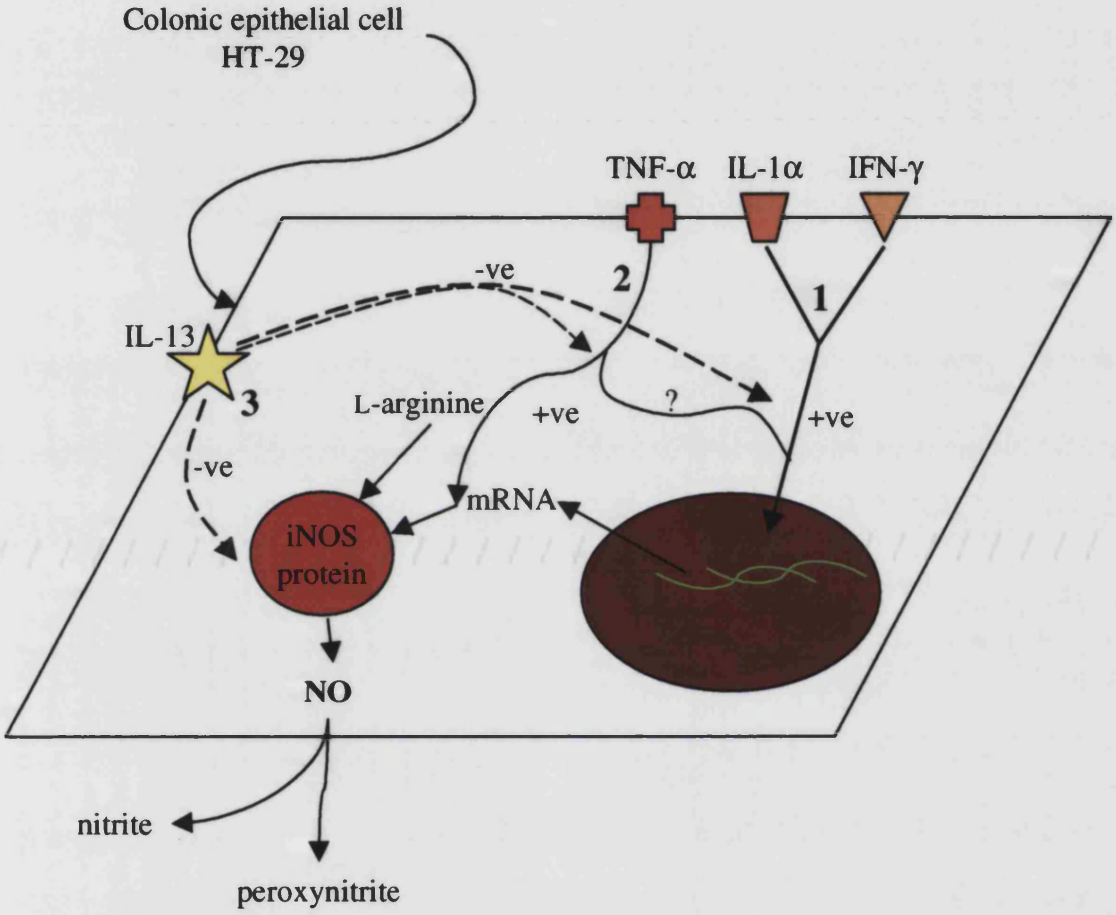


Figure 3.4. A model system for nitric oxide production by colonic epithelial cells. **1.** Pro-inflammatory cytokines, IL-1α and IFN-γ induce iNOS expression and activity. **2.** Further stimulation with TNF-α has a largely post-transcriptional effect, increasing nitrite output. **3.** An inhibitory effect on iNOS expression and activity is achieved by pre-treatment with the anti-inflammatory cytokine, IL-13. Pharmacological intervention is achievable at many points downstream of each cytokine's receptor, see text.

4. IL-13 SIGNAL TRANSDUCTION IN HT-29 CELLS

4.1 RESULTS

CALCIUM MOBILISATION

IL-13-induced inhibition of iNOS activity is not mediated by intracellular calcium mobilisation

Little is currently known about IL-13 signal transduction, so initial investigations were based on published data on IL-4 signalling and the little there was on IL-13 signalling. IL-4 had been shown to trigger a unique second messenger pathway in human B cells. This is characterised by a rapid, transient production of inositol (1,4,5)-trisphosphate and mobilisation of Ca^{2+}, followed after a brief lag period by an increase in intracellular cAMP (Finney *et al.*, 1990). This pathway had also been shown to be activated by IL-13 in human monocytes and required for the IL-13-mediated inhibition of protein kinase C-triggered respiratory burst (Sozzani *et al.*, 1995). However, IL-13 did not result in the mobilisation of Ca^{2+} ions in HT-29 cells (Fig. 4.1), as compared with bradykinin as a positive control. This suggests that IL-13 does not utilise this signalling mechanism to mediate its effects.

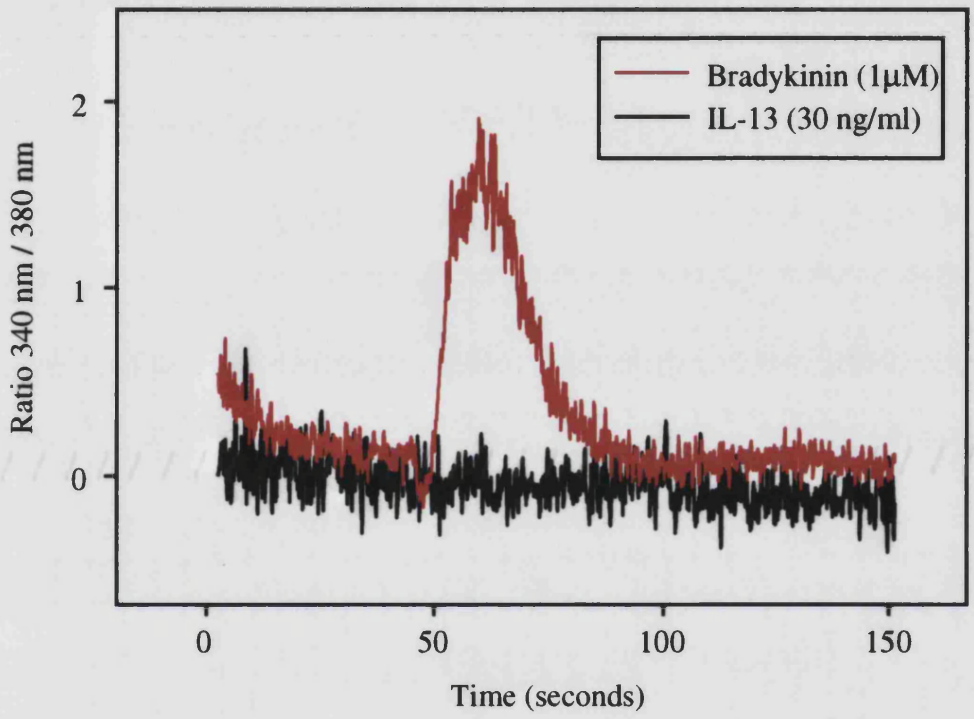


Figure 4.1. Growth-arrested HT-29 cells were loaded with fura-2/AM on glass coverslips and stimulated with bradykinin, as a positive control, and IL-13 (30 ng/ml). The $[Ca^{2+}]_i$ mobilisation was measured fluorometrically. The traces are representative of two separate experiments.

ACTIVATION OF PHOSPHATIDYLINOSITOL 3-KINASE BY IL-13

IL-13 enhances the in vitro lipid kinase activity of p85 immunoprecipitates

As IL-13 and IL-4 share many similarities with regard to function and signalling events (see section 1.4), it seemed likely that IL-13 might share the ability with IL-4 to activate PI 3-kinase (Gold *et al.*, 1994). In order to investigate this possibility, immunoprecipitates of the p85 regulatory subunit of PI 3-kinase were assayed for *in vitro* lipid kinase activity. Treatment of HT-29 cells with IL-13 (30 ng/nl) induced a transient increase in *in vitro* lipid kinase activity with maximum activity present in p85 immunoprecipitates derived from the 30 second time point (Fig. 4.2A). The p85-associated lipid kinase activity declined back to basal levels after 10 minutes stimulation with IL-13. Also, treatment with IL-13 (0.3 - 30 ng/ml) for 30 seconds resulted in a concentration-dependent increase in the *in vitro* lipid kinase activity present in p85 immunoprecipitates (Fig. 4.2B). The IL-13-induced increase in lipid kinase activity was also inhibited by 10 min pretreatment with wortmannin (10 - 300 nM), (Fig. 4.3A) and 15 min pretreatment with LY294002 (1 - 30 μM), (Fig. 4.3B). Taken together, these data have strongly identified PI 3-kinase as an important signalling enzyme activated by IL-13.

IL-13 induces the accumulation of D-3 phosphatidylinositol lipids

PI 3-kinase activation was also determined by measuring the accumulation of D-3 phosphatidylinositol lipids after stimulating ^{32}P-labelled HT-29 cells with IL-13 (example traces from the HPLC shown in Fig. 4.4). Treatment with IL-13 (30 ng/ml) resulted in a rapid and transient increase in the accumulation of PI (3,4,5) P_3 with maximum

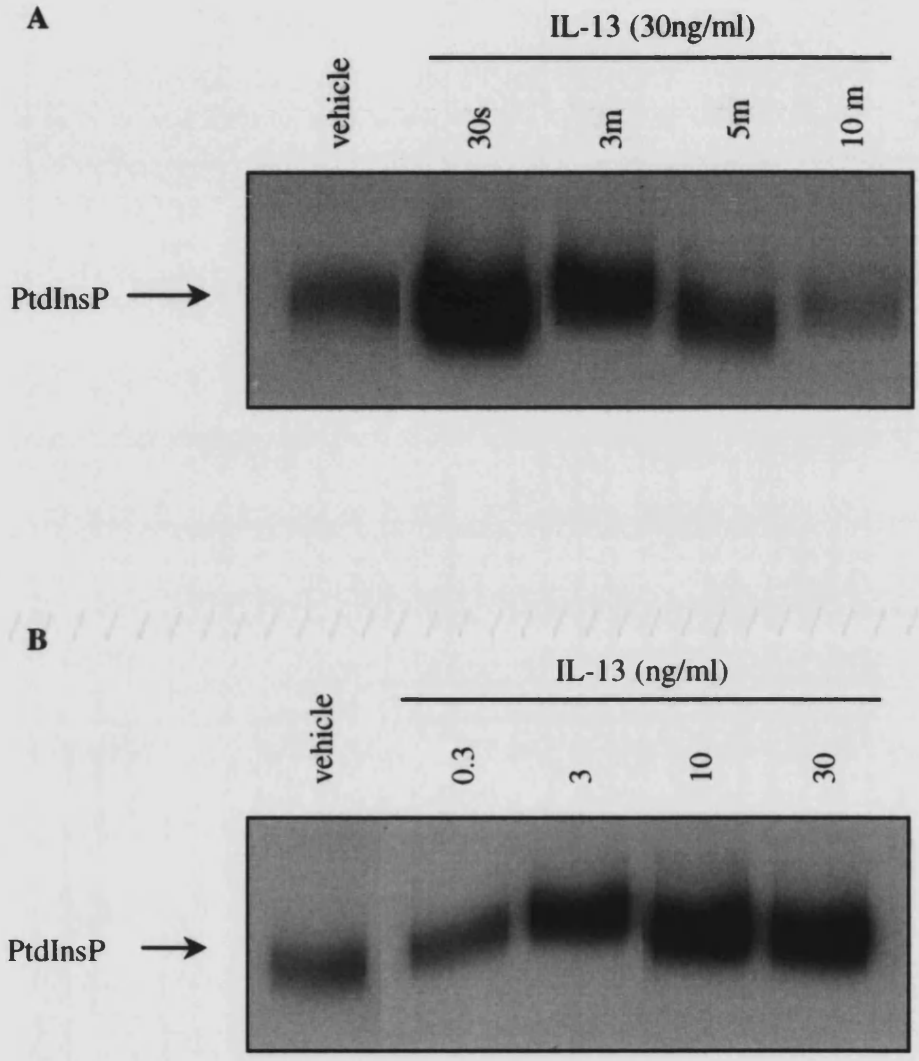


Figure 4.2. HT-29 cells (3.5 x 10^6) were A, stimulated with 30 ng/ml IL-13 for the times indicated and B, stimulated with IL-13 at 37 °C at the concentrations indicated for 30 seconds. Cells were lysed and subjected to immunoprecipitation with anti-p85 mAb and assayed for *in vitro* lipid kinase activity, as described in section 2.2.7.2. Data is representative of three experiments.

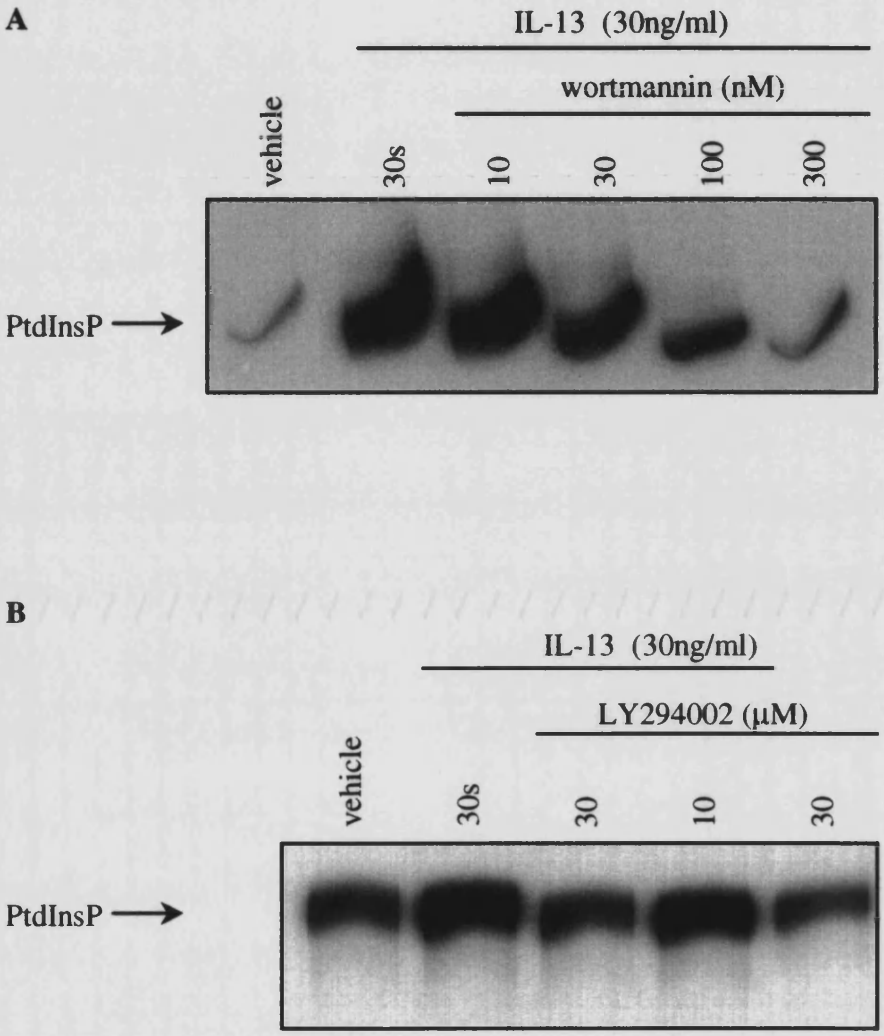


Figure 4.3. HT-29 cells (3.5 x 10^6) were A, incubated for 10 minutes with wortmannin or B, for 15 minutes with LY294002 at the concentrations indicated , followed by treatment with IL-13 (30 ng/ml) for 30 seconds at 37 °C. Cells were lysed and subjected to immunoprecipitation with anti-p85 mAb and assayed for *in vitro* lipid kinase activity, as described in section 2.2.7.2. Data is representative of three experiments.

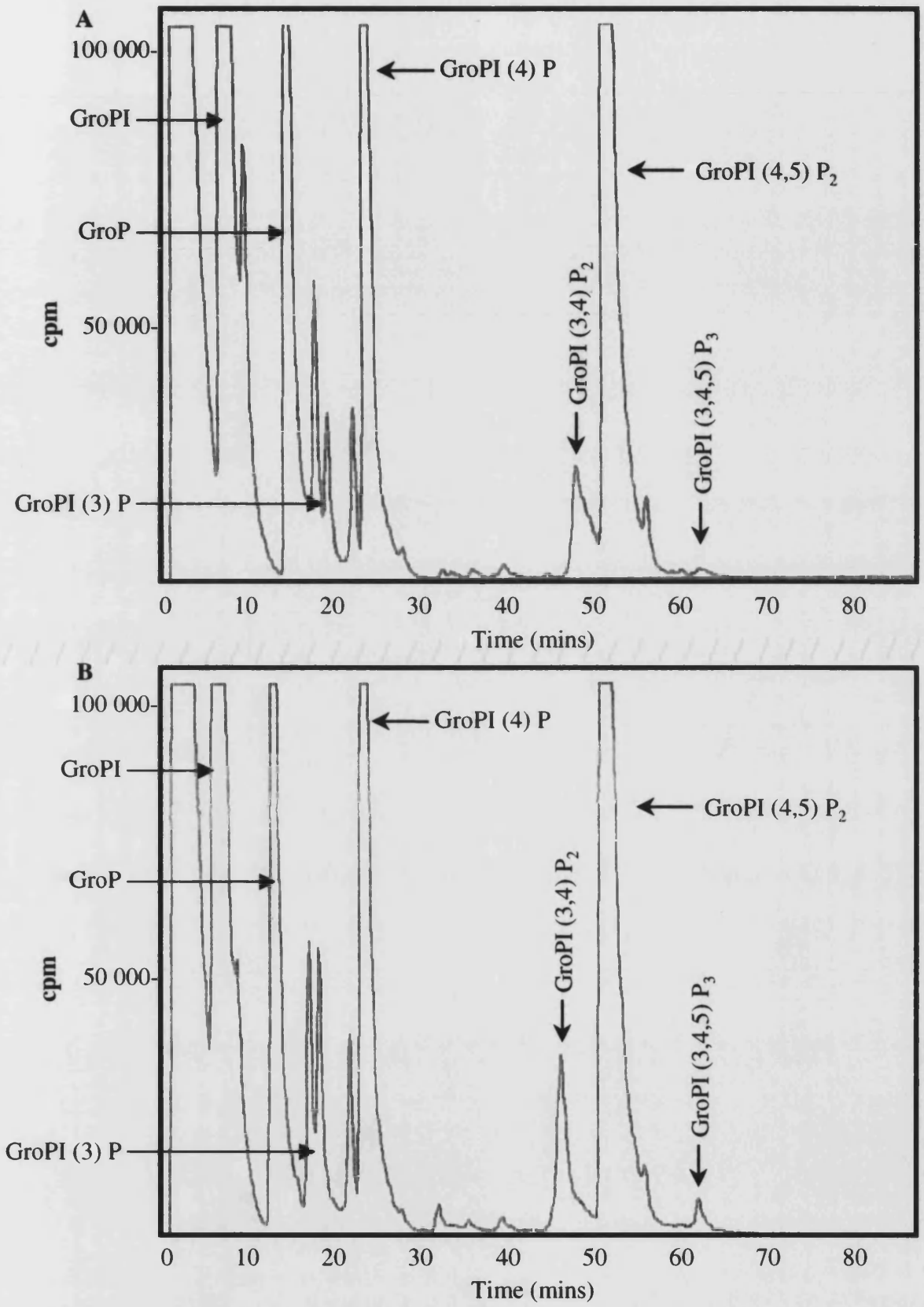


Figure 4.4. [^{32}P]Orthophosphate-labelled HT-29 (2 x 10^7 cells/120 µl) were A, stimulated with vehicle or B, stimulated with IL-13 (30 ng/ml) for 1 minute. PI lipids were extracted and deacylated, followed by HPLC separation. Data is representative of original traces before analysis.

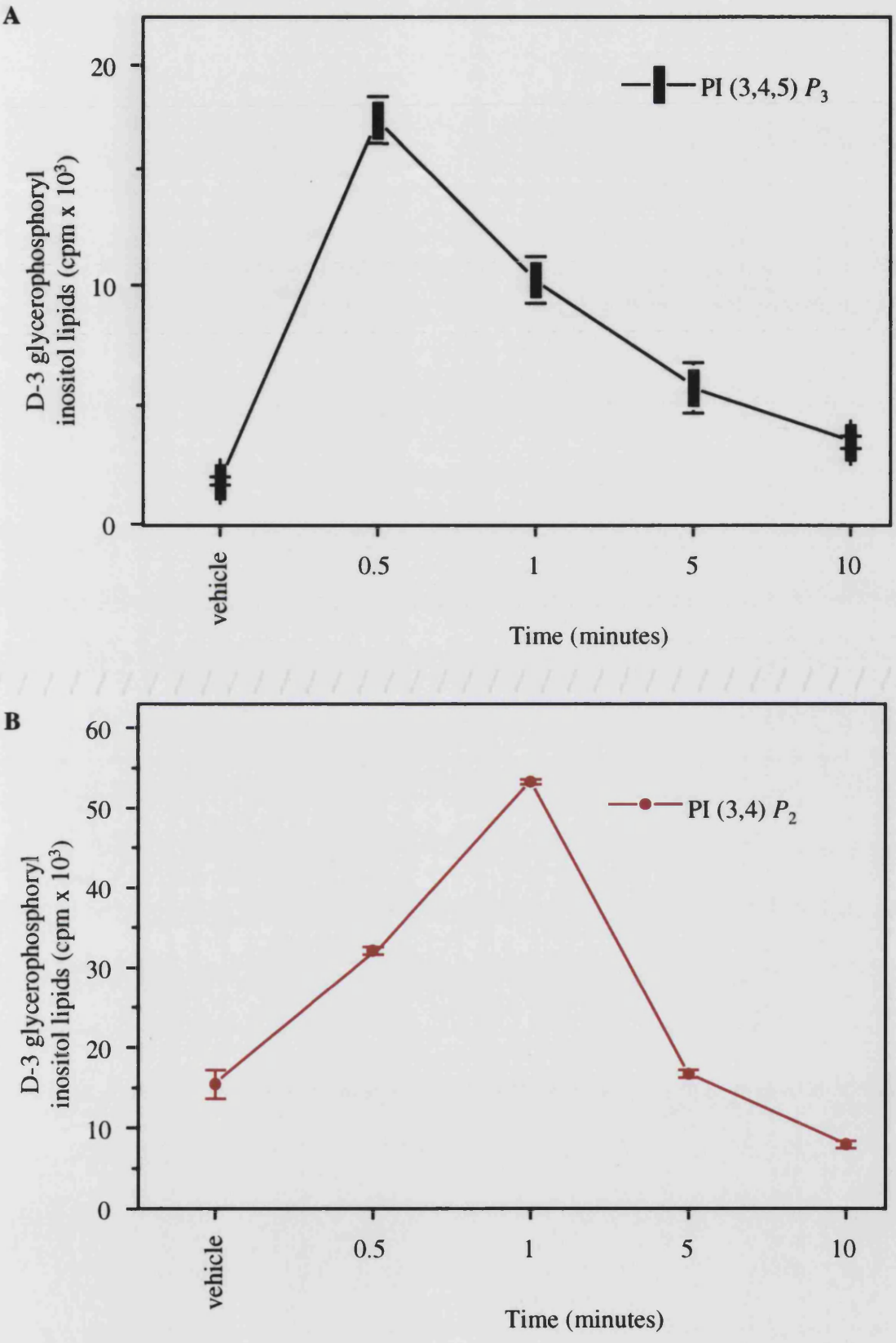


Figure 4.5. [^{32}P]Orthophosphate-labelled HT-29 (2 x 10^7 cells/120 µl) were stimulated with vehicle or IL-13 (30 ng/ml) for the times indicated. Phospholipids were extracted and deacylated, followed by HPLC separation and analysis as described in Section 2.2.7.1. A. PI (3,4,5) P_3 and B. PI (3,4) P_2 Data is the mean of two separate experiments, and is representative of three separate experiments in total.

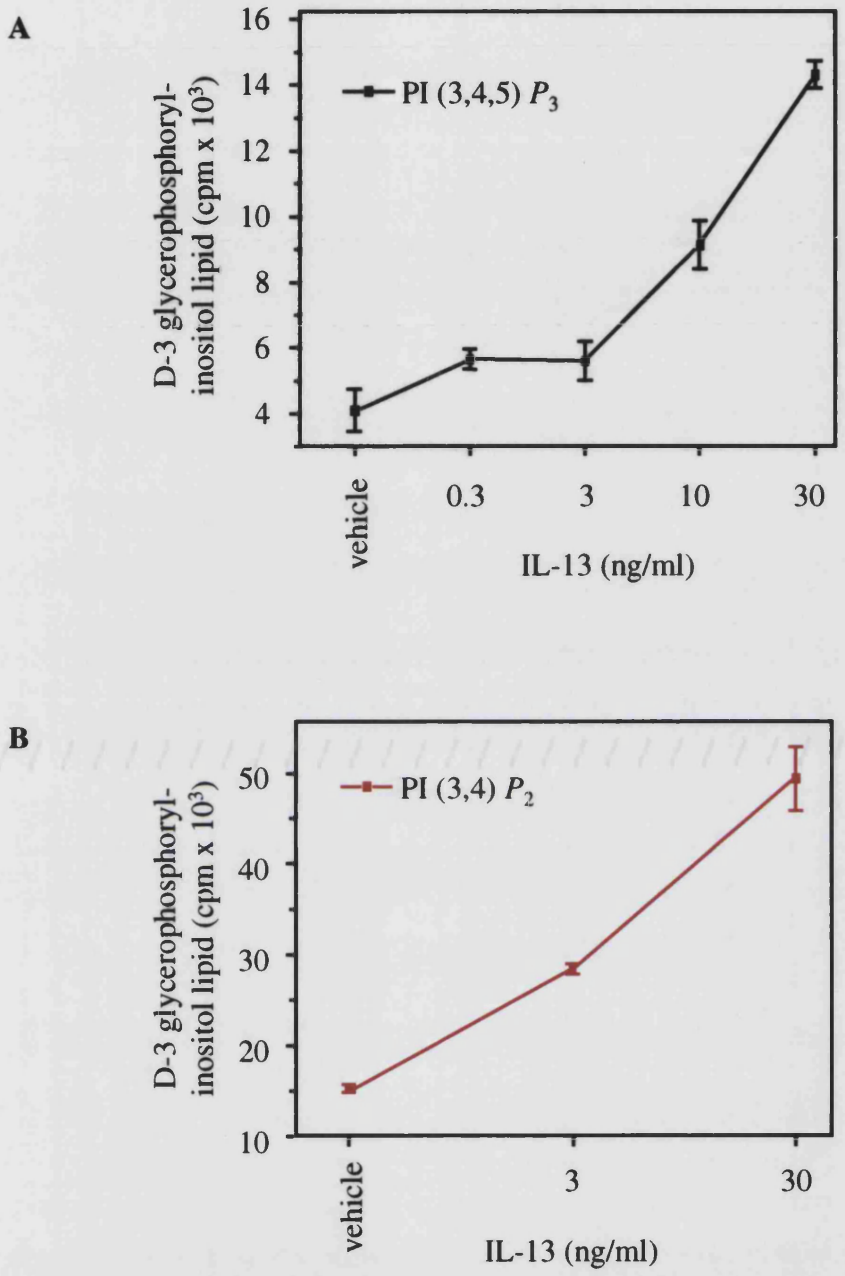


Figure 4.6. [^{32}P]Orthophosphate-labelled HT-29 (2 x 10^7 cells/120 µl) were stimulated with vehicle or IL-13 at the concentrations indicated for A, 30 seconds or B, for 1 minute. A, PI (3,4,5) P_3 and B, PI (3,4) P_2 were extracted and deacylated, followed by HPLC separation and analysis as described in Section 2.2.7.1. Data is the mean of two separate experiments, and is representative of three separate experiments in total.

accumulation occurring after 30 seconds (Fig. 4.5A). The levels of PI (3,4,5) P_3 had declined back to basal levels within 10 minutes of IL-13 treatment. In addition, IL-13 also induced the accumulation of another D-3 phosphatidylinositol lipid, namely PI (3,4) P_2 (Fig. 4.5B). However, the accumulation of PI (3,4) P_2 occurred with slower kinetics, such that maximum accumulation of PI (3,4) P_2 occurred at 1 minute post-IL-13 treatment (Fig. 4.5B), although the levels of PI (3,4) P_2 also declined back to basal levels within 10 minutes of stimulation. The apparent lag time for the accumulation of PI (3,4) P_2 compared with the accumulation of PI (3,4,5) P_3 is consistent with the proposal that PI (3,4) P_2 is the metabolic breakdown product of PI (3,4,5) P_3 (Stephens *et al.*, 1993).

The effect of IL-13 on D-3 PI accumulation was concentration-dependent. Hence, treatment of HT-29 cells with IL-13 (0.3 - 30 ng/ml) for 30 seconds resulted in the concentration-dependent accumulation of PI (3,4,5) P_3 (Fig. 4.6A). Equally, treatment with IL-13 (3 and 30 ng/ml) for 1 minute resulted in the concentration-dependent accumulation of PI (3,4) P_2 (Fig. 4.6B). Moreover, the accumulation of PI (3,4,5) P_3 (Fig. 4.7A and 4.8A) and PI (3,4) P_2 (Fig. 4.7B and 4.8B) after IL-13 treatment was inhibited by pretreatment with the PI 3-kinase inhibitors, wortmannin (0.5 nM - 1μM) and LY294002 (1 - 30 μM) in a concentration-dependent manner.

IL-13 INDUCES PHOSPHORYLATION OF CELLULAR PROTEINS

IL-13 induces tyrosine phosphorylation of IRS-1 and association with p85

To define more clearly the mechanism of interaction of the IL-13R with PI 3-kinase, experiments were performed to investigate whether or not the coupling of the IL-13R to PI

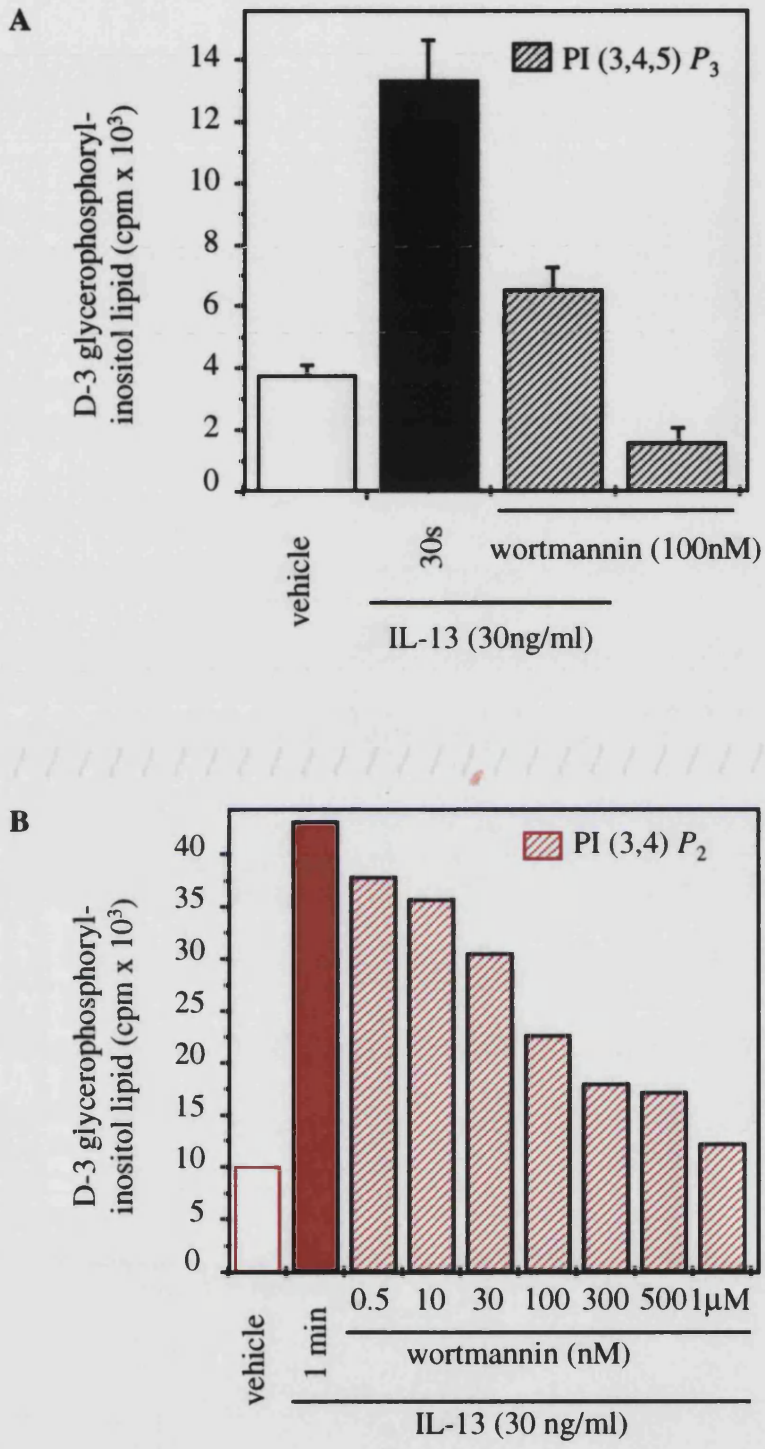


Figure 4.7. [^{32}P]Orthophosphate-labelled HT-29 (2 x 10^7 cells/120 μl) were stimulated with vehicle or wortmannin at the concentrations indicated for 10 minutes, followed by stimulation with either vehicle or IL-13 (30 ng/ml) for A, 30 seconds or B, for 1 minute. A, PI (3,4,5) P_3 and B, PI (3,4) P_2 were extracted and deacylated, followed by HPLC separation and analysis as described in Section 2.2.7.1. This is representative data.

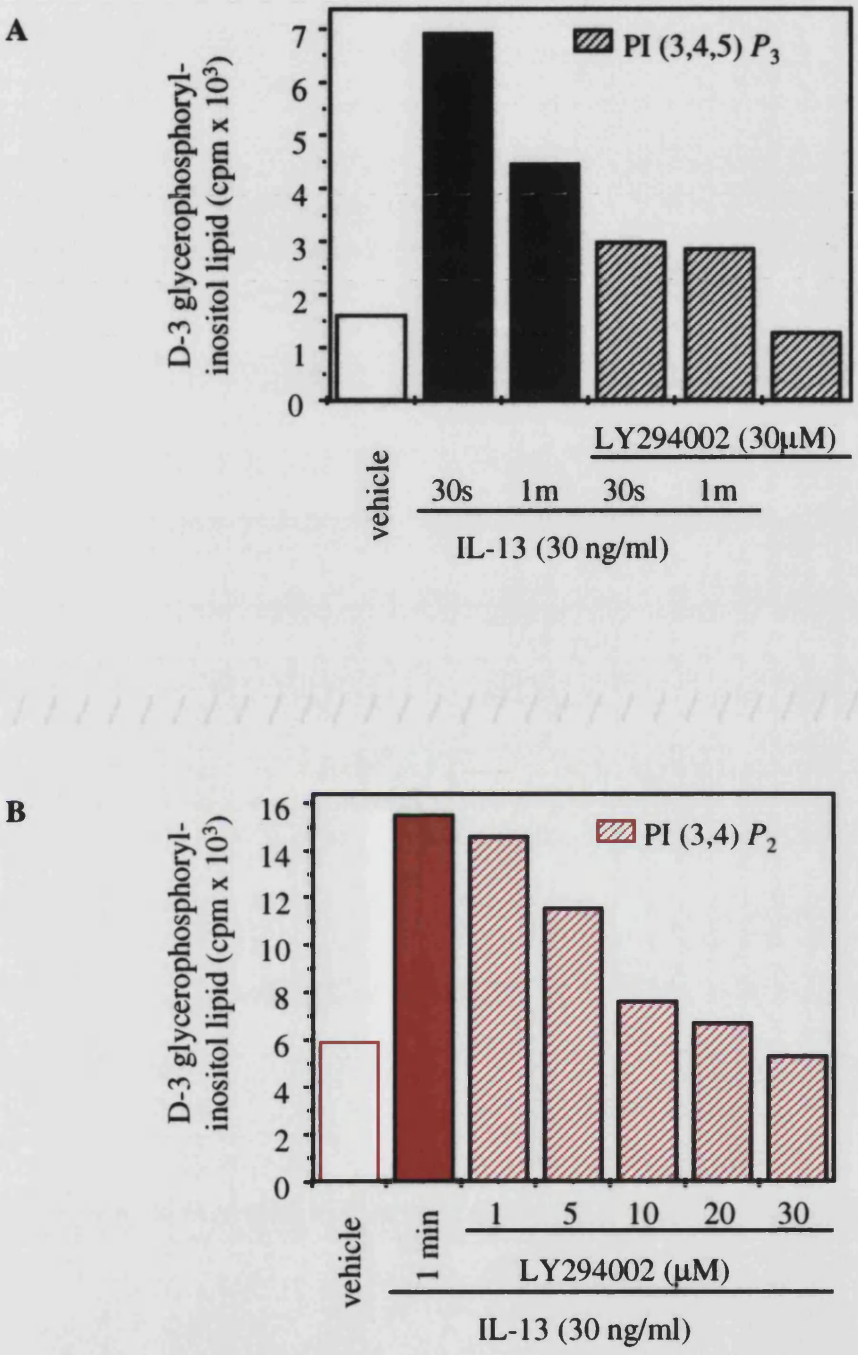


Figure 4.8. [^{32}P]Orthophosphate-labelled HT-29 (2 x 10^7 cells/120 μl) were stimulated with vehicle or LY294002 at the concentrations indicated for 15 minutes followed by stimulation with either vehicle or IL-13 (30 ng/ml) for A, 30 seconds or B, for 1 minute. A, PI (3,4,5) P_3 and B, PI (3,4) P_2 were extracted and deacylated, followed by HPLC separation and analysis as described in Section 2.2.7.1. This is representative data.

3-kinase involved the adapter molecules IRS-1 and/or IRS-2. Both IRS-1 and IRS-2 have previously been implicated in IL-4R- and IL-13R-mediated signal transduction events (Welham *et al.*, 1995; Lefort *et al.*, 1995; Keegan *et al.*, 1994 and Schnyder *et al.*, 1996). Both can associate with PI 3-kinase via specific phosphotyrosine-containing sequences that bind the p85 subunit (Sun *et al.*, 1995 and Sun *et al.*, 1991). Accordingly, immunoprecipitations were performed on cell lysates derived from IL-13-stimulated HT-29 cells using antibodies directed against either the p85 subunit of PI 3-kinase, IRS-1 or IRS-2 (Fig. 4.9). The resulting precipitates were immunoblotted with the 4G10 anti-phosphotyrosine mAb. Two tyrosine-phosphorylated proteins migrating at 165 and 170 kDa, consistent with the molecular weights of IRS-1 and IRS2, respectively, were co-immunoprecipitated by the p85 mAb (Fig. 4.9A). Immunoblotting of the p85 immunoprecipitates with the polyclonal anti-IRS-1 revealed a time-dependent increase in the association of IRS-1 with p85 that correlated with the kinetics of tyrosine phosphorylation of the proteins migrating at 165-170 kDa (Fig. 4.9A). Furthermore, immunoblotting of the IRS-1 immunoprecipitates from IL-13-stimulated HT-29 cells with the 4G10 anti-phosphotyrosine mAb confirmed that IL-13 stimulation induced the strong tyrosine phosphorylation of IRS-1 (Fig. 4.9B). However, similar experiments using IRS-2 immunoprecipitates (Fig. 4.9C) revealed no detectable increase in IRS-2 tyrosine phosphorylation after IL-13 stimulation, although there was some basal phosphorylation of this protein. Each blot was stripped and reprobed with anti-p85 (Fig. 4.9A), anti-IRS-1 (Fig. 4.9B) and anti-IRS-2 (Fig. 4.9C) to confirm efficiency of immunoprecipitation in each case.

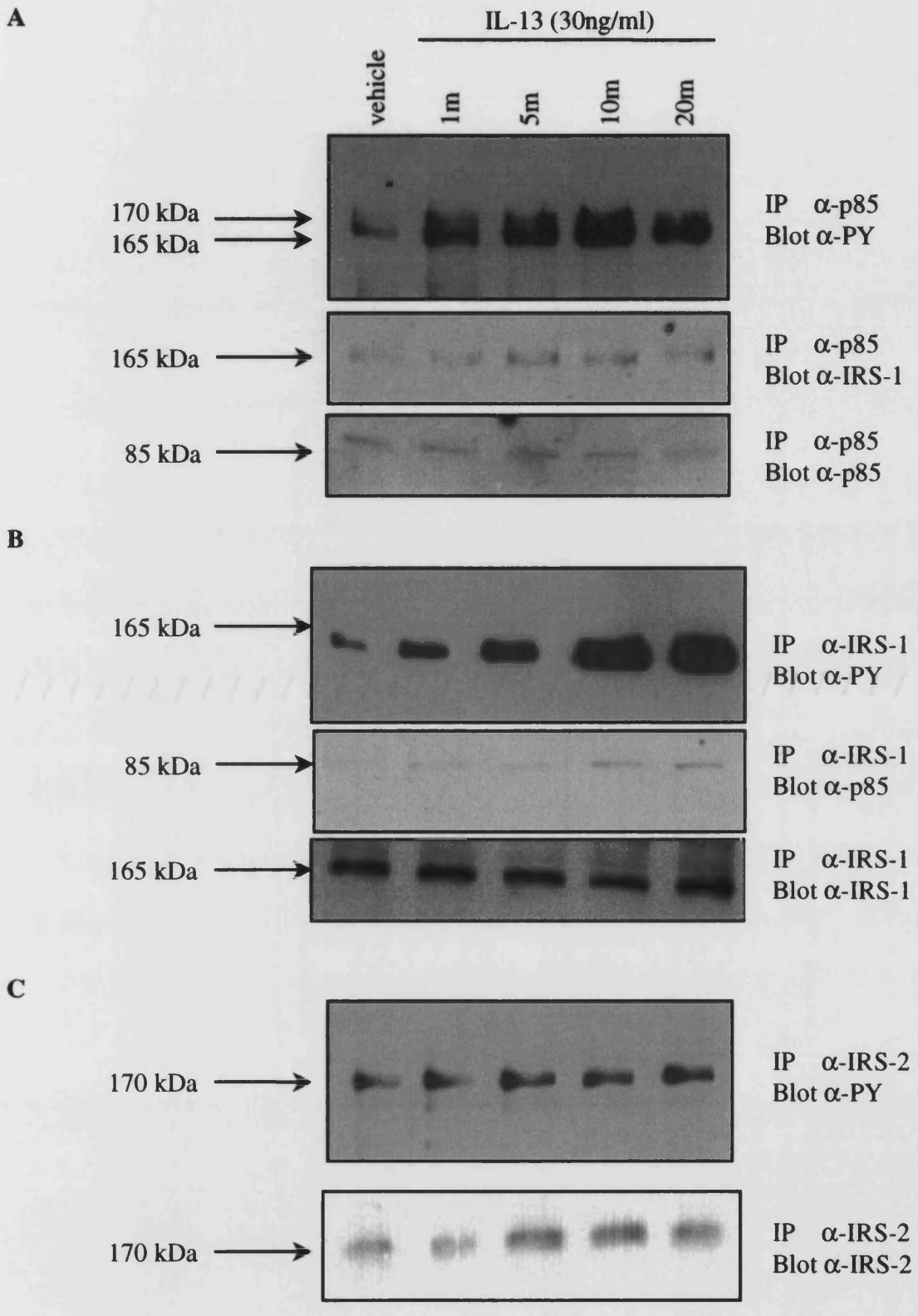


Figure 4.9. HT-29 cells (3.5×10^6) were stimulated with IL-13 (30 ng/ml) at 37 °C for the times indicated. Cells were lysed, subjected to immunoprecipitation with A, α-p85 mAb, B, α-IRS-1 and C, α-IRS-2 and subjected to Western analysis Nitrocellulose membranes were immunoblotted with the antiphosphotyrosine antibody, 4G10, after which they were stripped and reprobed with the antibodies indicated. Each blot is representative of at least three separate experiments.

PI 3-kinase inhibitors prevent IL-13 suppression of pro-inflammatory cytokine-induced iNOS expression and nitrite production

As shown previously (Kolios *et al.*, 1995) and results from the previous section, growth-arrested HT-29 monolayers stimulated with the IL-1α/IFN-γ/TNF-α combination of pro-inflammatory cytokines result in the optimal generation of nitrite (Fig. 4.10A) and expression of iNOS mRNA (Fig. 4.10B). Both functional events can be inhibited by pretreatment with IL-13 (30 ng/ml) for 1 hour before the addition of the cytokine mixture. However, the addition of wortmannin (10 - 300 nM) to HT-29 cells 10 minutes before IL-13 treatment prevents the inhibitory effects of IL-13 on iNOS mRNA expression (Fig. 4.10B) and nitrite production (Fig. 4.10A). Similarly, the addition of the structurally unrelated LY294002 (1 – 30 μM) for 15 minutes before IL-13, resulted in a dose-dependent reversal of the IL-13 inhibition of iNOS transcripts (Fig. 4.11B) and nitrite production (Fig. 4.11A). Taken together, these results strongly suggest a role for the signalling enzyme, PI 3-kinase, in the transduction of the IL-13-induced signal, which down-regulates the iNOS gene.

4.2 DISCUSSION

It has been shown that IL-13 activates PI 3-kinase in a human colonic epithelial cell line as determined by the accumulation of D-3 PI lipids from intact cells and increased lipid kinase activity present in immunoprecipitates of the p85 regulatory subunit of PI 3-kinase. In addition, IL-13 stimulates the tyrosine phosphorylation of the adapter molecule IRS-1, which has previously been reported to facilitate receptor coupling to PI 3-kinase (Sun *et al.*, 1991). This is the first evidence that IL-13 can stimulate the increased lipid kinase activity

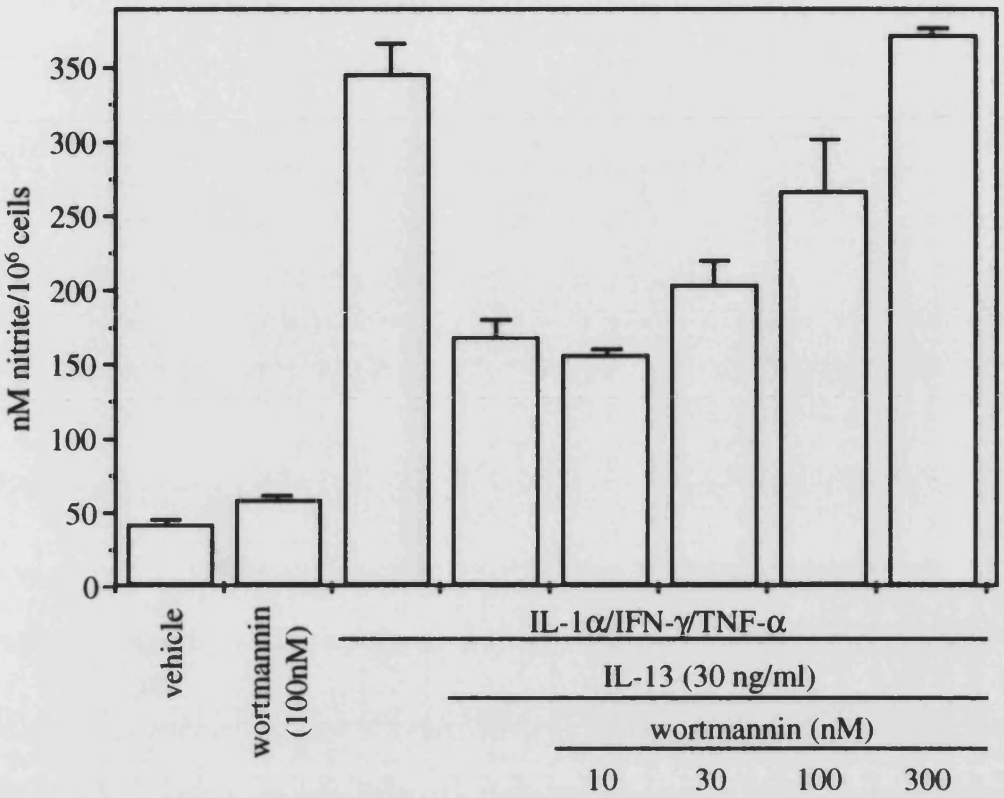


Figure 4.10A. Growth-arrested HT-29 monolayers were treated with vehicle or wortmannin (nM) for 10 minutes prior to the addition of IL-13 for 1 hour, followed by the cytokines, as indicated. After 24 hours, nitrite in the supernatants was measured using the fluorometric nitrite assay. Data is the mean ± SEM, n = 3.

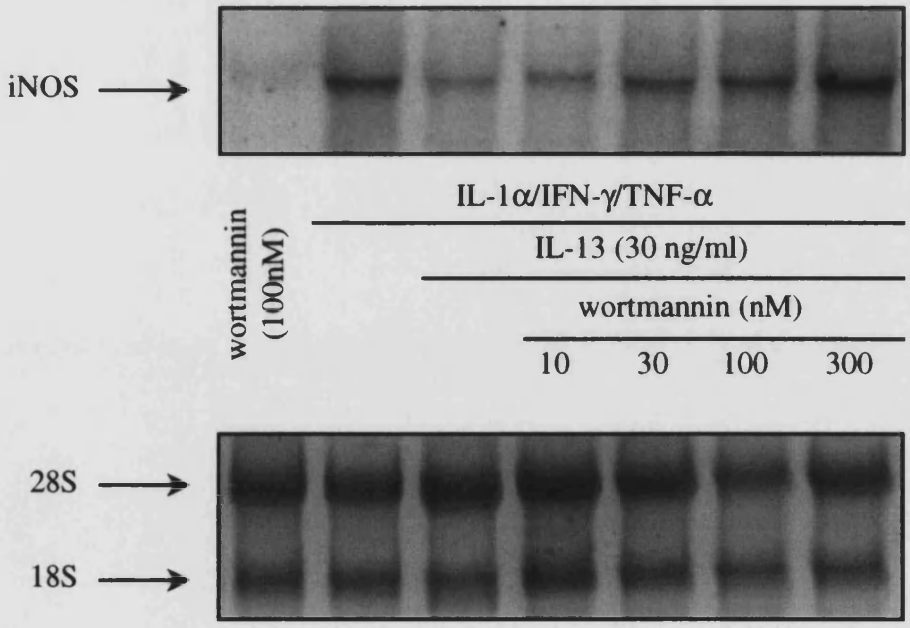


Figure 4.10B. Growth-arrested HT-29 monolayers were treated with wortmannin (nM) for 10 minutes prior to the addition of either vehicle or IL-13 for 1 hour, followed by cytokines, as indicated. After 24 hours, cells were harvested for Northern analysis. The top panel shows the iNO mRNA detected and the bottom panel verifies equal loading. Data shown is representative of three experiments.

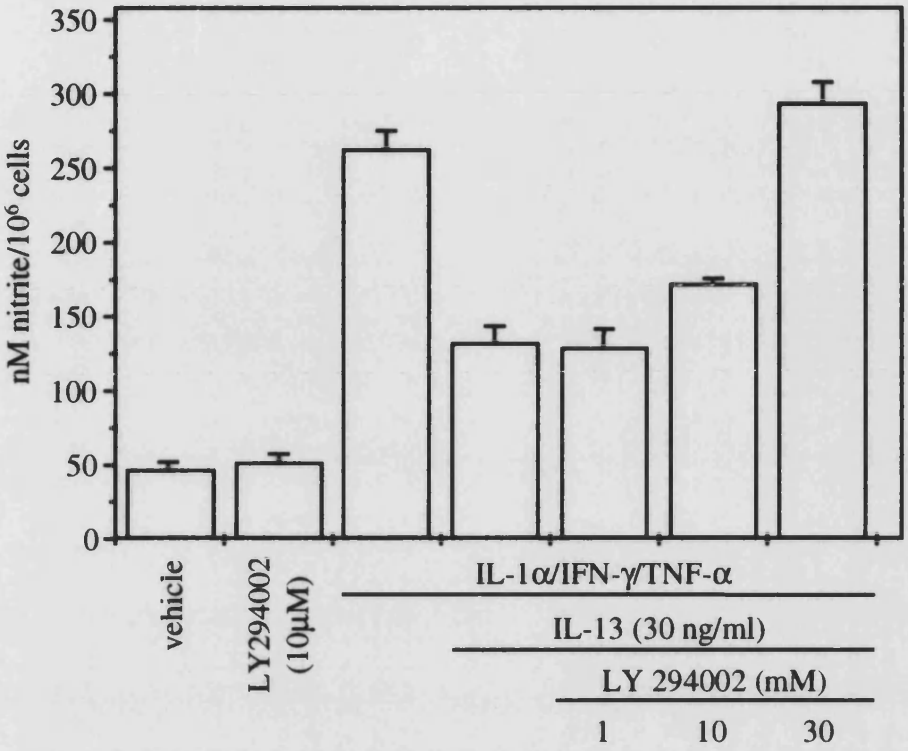


Figure 4.11A. Growth-arrested HT-29 monolayers were treated with LY294002 (μM) for 15 minutes prior to the addition of IL-13 for 1 hour, followed by the cytokines, as indicated. After 24 hours, nitrite in the supernatants was measured using the fluorometric nitrite assay. Data is the mean ± SEM, n = 3.

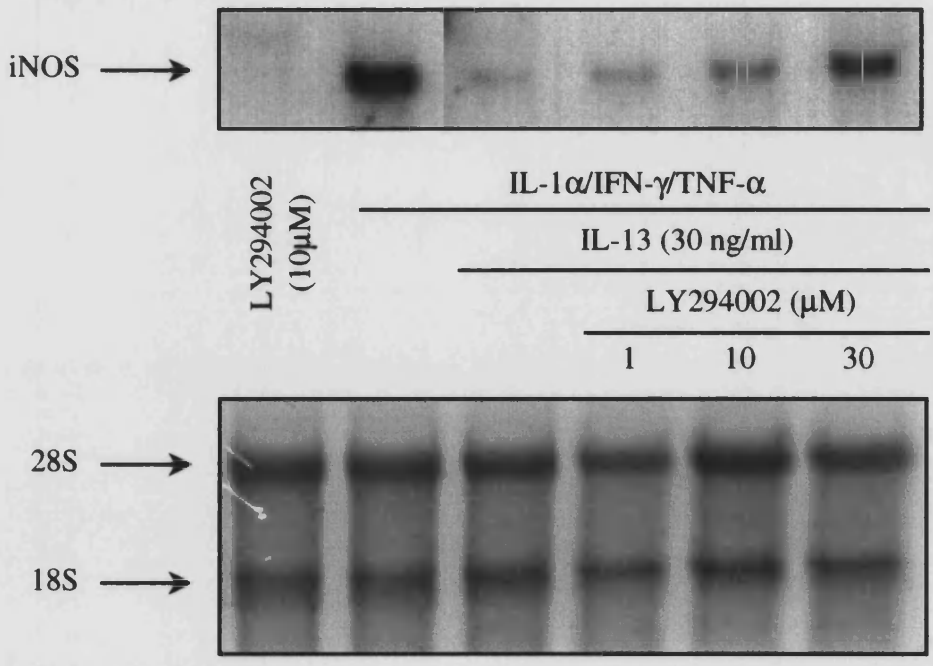


Figure 4.11B. Growth-arrested HT-29 monolayers were treated with LY294002 (μM) for 15 minutes prior to the addition of either vehicle or IL-13 for 1 hour, followed by cytokines, as indicated. After 24 hours, cells were harvested for Northern analysis. The top panel shows the iNOS mRNA detected and the bottom panel verifies equal loading. Data is representative of two experiments

of PI 3-kinase and is an important observation given that D-3 PI lipids are increasingly thought to act as important regulatory molecules utilised by a plethora of receptors involved in diverse outcomes (see section 1.4).

Accordingly, the two structurally unrelated PI3-K inhibitors, wortmannin and LY294002, have been used to demonstrate the IL-13-mediated activation of PI 3-kinase. The resulting accumulation of D-3 PI lipids has also been shown necessary for the IL-13-induced suppression of iNOS mRNA expression and nitrite production induced by the mixture of pro-inflammatory cytokines IL-1α, IFN-γ and TNF-α. The ability of IL-13 to inhibit nitric oxide production and iNOS activity in macrophages (Doyle *et al.*, 1994 and Doherty *et al.*, 1994), mesangial cells (Saura *et al.*, 1996) and now HT-29 carcinoma cells (this work) induced by pro-inflammatory cytokines seems to be an important function of IL-13. Until now, however, the signalling cascades that mediate the effects of IL-13 on iNOS have not previously been characterised. This demonstration that PI 3-kinase inhibitors prevent the effects of IL-13 on iNOS is similar to a previous report that demonstrated that insulin-induced inhibition of phosphoenolpyruvate carboxykinase is dependent on PI 3-kinase activation (Gabbay *et al.*, 1996). This further demonstrates an important role for PI 3-kinase in the negative regulation of the induction of specific mRNA (Reif *et al.*, 1997).

To date, several forms of mammalian PI 3-kinase have been identified, and these include various isoforms of the p85/p110 heterodimer (Otsu *et al.*, 1991), The G protein-coupled PI 3-kinase γ (Stoyanov *et al.*, 1995 and the PtdIns-specific 3-kinase (Volinia *et al.*, 1995). The p85/p110 heterodimer is the best studied of these lipid kinases, and reagents to either p85 or p110 are readily available and reliable. In contrast, studies relating to PI 3-kinase γ and PtdIns 3-kinase are hampered by a lack of reliable and commercially available

antibodies. *In vitro* assays for associated lipid kinase activity revealed that IL-13 treatment increased the amount of lipid kinase activity present in p85α immunoprecipitates. These data strongly indicate that the p85/p110 heterodimeric PI 3-kinase is coupled to and activated by the IL-13R. However, coupling of the IL-13R to other PI 3-kinase family members cannot be entirely discounted, and these lipid kinases may potentially contribute to the production of total PI (3,4,5) P_3 extracted from IL-13-stimulated ^{32}P-labelled HT-29 cells. Although the precise nature of the IL-13R is unclear, the known p85 binding motifs have not so far been identified in the intracellular domain of the related IL-4R. Nevertheless, the IL-4R has been demonstrated to associate with and/or activate PI 3-kinase (Izuhara *et al.*, 1993; Gold *et al.*, 1994 and Wang *et al.*, 1992). This coupling is thought to occur via an intermediate adapter such as IRS-2, which does contain several YXXM motifs (Sun *et al.*, 1995). IRS-2 is tyrosine phosphorylated after IL-4 treatment, and this phosphorylation may be required for association of IRS-2 with PI 3-kinase (Sun *et al.*, 1995). IRS-2 has also been demonstrated to be tyrosine phosphorylated and to associate with PI 3-kinase after IL-13 treatment (Welham *et al.*, 1995 and Lefort *et al.*, 1995), suggesting that IRS-2 may also facilitate the coupling of the IL-13R to the putative signalling cascades regulated by PI 3-kinase. Interestingly, this study revealed that IL-13 was not able to induce detectable tyrosine phosphorylation of IRS-2 in HT-29 cells, although it did stimulate a rapid and strong tyrosine phosphorylation of IRS-1 within 1 minute post-stimulation. Generally, the kinetics of IL-13-stimulated IRS-1 tyrosine phosphorylation correlated with the kinetics of IL-13-stimulated PI 3-kinase activation. Data indicate that IRS-1 may potentially have an important role in coupling the IL-13R to PI 3-kinase in HT-29 cells. However, a role for IRS-2 in the coupling of IL-13R to PI 3-kinase in HT-29 cells cannot be entirely ruled out because low stoichiometric tyrosine phosphorylation of IRS-2 after IL-13 stimulation may be sufficient to allow IRS-2 to recruit PI 3-kinase to the IL-13R.

This demonstration that IL-13 differentially stimulates the tyrosine phosphorylation of IRS-1, but not IRS-2, correlates with previous observations that IRS-1 is a major phosphoprotein of non-haematopoietic cells. IRS-2 phosphorylation is induced principally in murine haematopoietic cell types by various growth factors and cytokines (Keegan *et al.*, 1994a; Sun *et al.*, 1992; Keegan *et al.*, 1994b; Morla *et al.*, 1988; Wang *et al.*, 1993 and Welham *et al.*, 1997). Other evidence exists to indicate considerable heterogeneity in both receptor structure and signal transduction in different cell types for both IL-4 and IL-13. For instance, in immune cells IL-4 is a growth and differentiation factor, and the common γ chain is associated with the IL-4R (Callard *et al.*, 1996). However, the common γ chain is not expressed in colon carcinoma cells in which IL-4 mediates a growth inhibitory effect (Murata *et al.*, 1996a). Moreover, both IL-4 and IL-13 induce the tyrosine phosphorylation of JAK2 in colon carcinoma cells (Murata *et al.*, 1996a and Murata *et al.*, 1996b). Neither are able to tyrosine phosphorylate JAK2 in immune cells (Welham *et al.*, 1995; Murata *et al.*, 1996a; Yin *et al.*, 1994; Witthuhn *et al.*, 1994 and Malabarba *et al.*, 1995). Similarly, IL-4 is unable to phosphorylate JAK3 in colon carcinoma cells (Murata *et al.*, 1996a), but is able to phosphorylate JAK3 in immune cells (Welham *et al.*, 1995; Yin *et al.*, 1994 and Malabarba *et al.*, 1995). In addition to phosphorylation of cellular proteins, IL-4 has been shown to trigger a unique second messenger pathway in human, but not mouse, B cells. This is characterised by a rapid, transient production of inositol (1,4,5)-trisphosphate and mobilisation of Ca^{2+}, followed after a brief lag period by an increase in intracellular cAMP (Finney *et al.*, 1990). This pathway is also activated by IL-13 in human monocytes and is required for the IL-13-mediated inhibition of protein kinase C-triggered respiratory burst (Sozzani *et al.*, 1995). It is interesting to note, however, that no detectable changes in intracellular Ca^{2+} concentration occurred in HT-29 cells after IL-13 stimulation. The

diversity and heterogeneity of both receptors and signalling pathways activated by IL-4 and IL-13 may therefore facilitate the differential functional effects of these related cytokines. Given the possible heterogeneity of signal transduction pathways coupled to the IL-13R, it will be necessary to determine whether IL-13 is able to activate PI 3-kinase in other cell types such as immune cells and to investigate the relevance of PI 3-kinase to other IL-13 functional events. This will undoubtedly help in evaluating the potential of the PI 3-kinase pathway as new and specific therapeutic target for intestinal inflammation.

In summary, IL-13 activates PI 3-kinase via the recruitment of IRS-1. In conclusion, this study provides the first evidence that PI 3-kinase activation is an important signal in determining the IL-13R-mediated inhibition of iNOS mRNA expression in response to inflammatory cytokines in the epithelial cell line HT-29 (Figure 4.12). Additional work is necessary to determine the precise downstream events that occur after PI 3-kinase activation in this system and will be addressed in the next section.

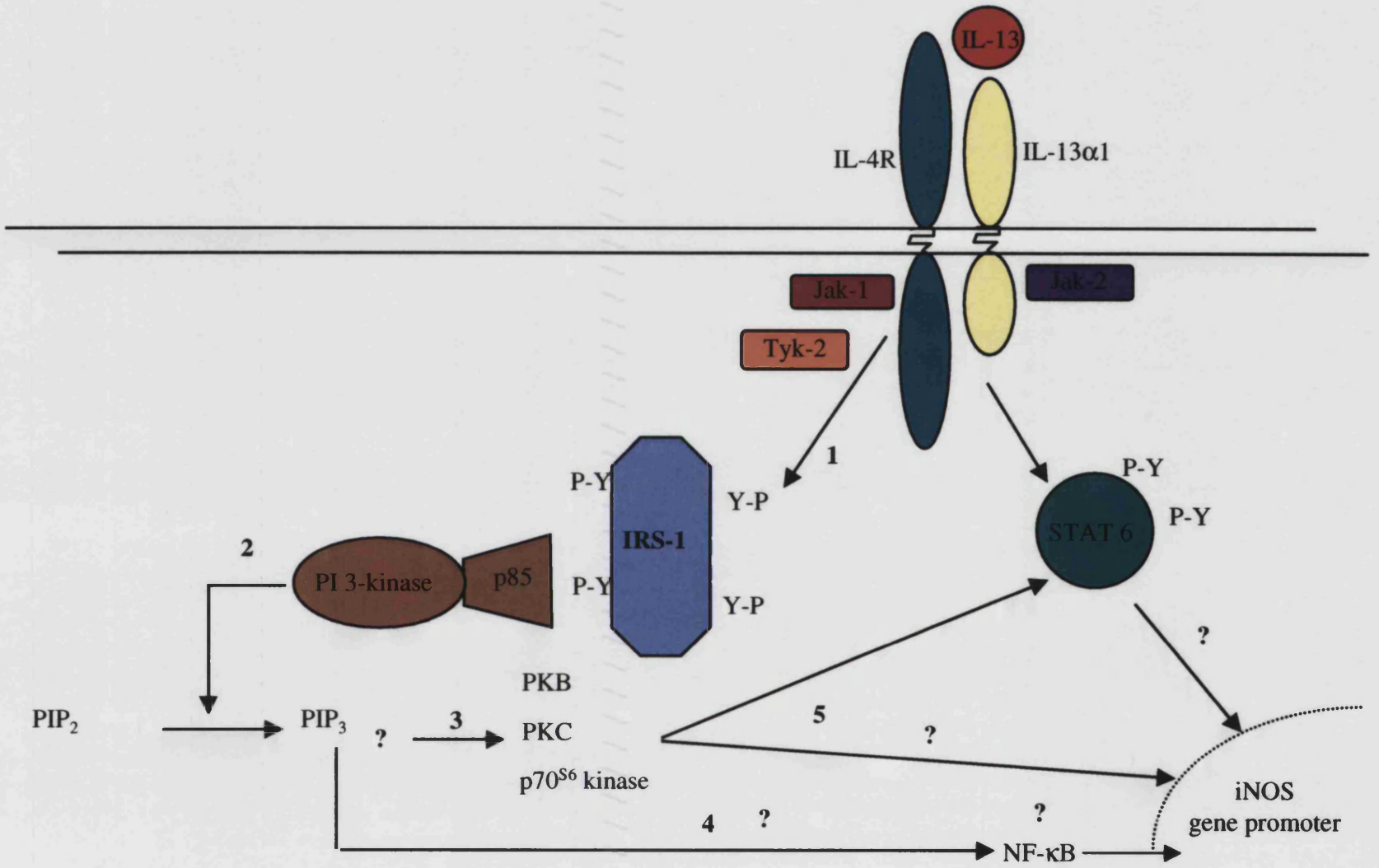


Figure 4.12. IL-13 mediated signalling events in HT-29 cells. IL-13 recruits IRS-1 (1) and activates PI 3-kinase (2), which leads to the formation of the signalling products, PI (3,4) P_2 and PI (3,4,5) P_3. Potential downstream targets include protein kinases B and C, as well as p70 S6-kinase (3). PI (3,4,5) P_3. Could have a direct effect on transcription factors, such as STAT6 and NF-κB (4) or an indirect effect mediated by activated downstream targets (5).

5. INDUCTION AND MODULATION OF APOPTOSIS IN HT-29 CELLS

As previously shown in section 3, a combination of pro-inflammatory cytokines (IL-1α/IFN-γ/TNF-α) up-regulates iNOS expression and generates NO in a human colonic epithelial cell line HT-29 (Kolios *et al.*, 1995). Also, it has recently been shown that IFN-γ, in combination with TNF-α or anti-CD95, induces apoptosis in HT-29 cells (Abreu-Martin *et al*, 1998) and an increased frequency of epithelial apoptosis mediated by the CD95-CD95L system is seen in ulcerative colitis (UC) (Sträter *et al.*, 1997 and Iwamoto *et al.*, 1996). However, colonic epithelial cell injury, resulting in impaired barrier function, could contribute to the pathogenesis of IBD (Gardiner *et al.*, 1998).

It has been postulated that overproduction of nitric oxide (NO) by inflamed mucosa may play a role in the pathophysiology of IBD due to the increased expression of the inducible form of nitric oxide synthase (iNOS) found in biopsies taken from patients with active UC as compared with normal colon (Kolios *et al.*, 1998). The production of NO might play a critical role in the resolution of inflammation (McCafferty *et al.*, 1997), possibly by inducing apoptosis in the leukocytic population recruited to the area (eg., neutrophils) (McCafferty *et al.*, 1997). Whilst NO has also been reported to inhibit apoptosis in several settings (Dimmeler *et al.*, 1997; Genaro *et al.*, 1995; Mannick *et al.*, 1997 and Kim *et al.*, 1997), it has also been reported to mediate cell death through mechanisms consistent with apoptosis in various cells including peritoneal macrophages (Messmer *et al.*, 1996; Albina *et al.*, 1993 and Sarih *et al.*, 1993), β-cells (Kaneto *et al.*, 1995 and Ankarcrona *et al.*, 1994) and thymocytes (Fehsel *et al.*, 1995).

Overproduction of NO may lead to oxidant-induced injury of the colon epithelial crypt (McKenzie *et al.*, 1996), possibly by the reaction with superoxide to form peroxynitrite which in turn results in the nitration of proteins on tyrosine residues (Singer *et al.*, 1996).

In this study, we sought to ascertain whether there is a relationship between NO production and apoptosis of HT-29 epithelial cells observed in response to a combination of cytokines and/or CD95 ligation. In addition, given that the ability of IL-13 to inhibit iNOS expression and NO generation in this system is driven by PI 3-kinase-dependent pathway, we investigated whether IL-13 could provide a cell survival signal through PI 3-kinase to protect against cytokine-driven apoptotic signals.

5.1 RESULTS

Apoptosis of colon epithelial cells induced by pro-inflammatory cytokines is inhibited by IL-13

Initial investigations took the form of determining the cytokine requirements for the induction of cell death. Growth arrested HT-29 monolayers stimulated with a combination of the pro-inflammatory cytokines IL-1α (10 ng/ml), IFN-γ (300 U/ml)) and TNF-α (100 ng/ml), resulted in a 25-80% increase in the expression of apoptotic markers over a time course of 4-24 hours (Table 5.1 and Fig. 5.1). This was assessed by a number of assays which measure early and late stage markers of apoptotic events, namely DNA fragmentation (Fig. 5.1A), externalisation of phosphatidylserine (Fig. 5.1B) and DNA-histone-association (Fig. 5.1C). In contrast, treatment with individual cytokines was insufficient to increase cell death above basal levels (Table 5.1).

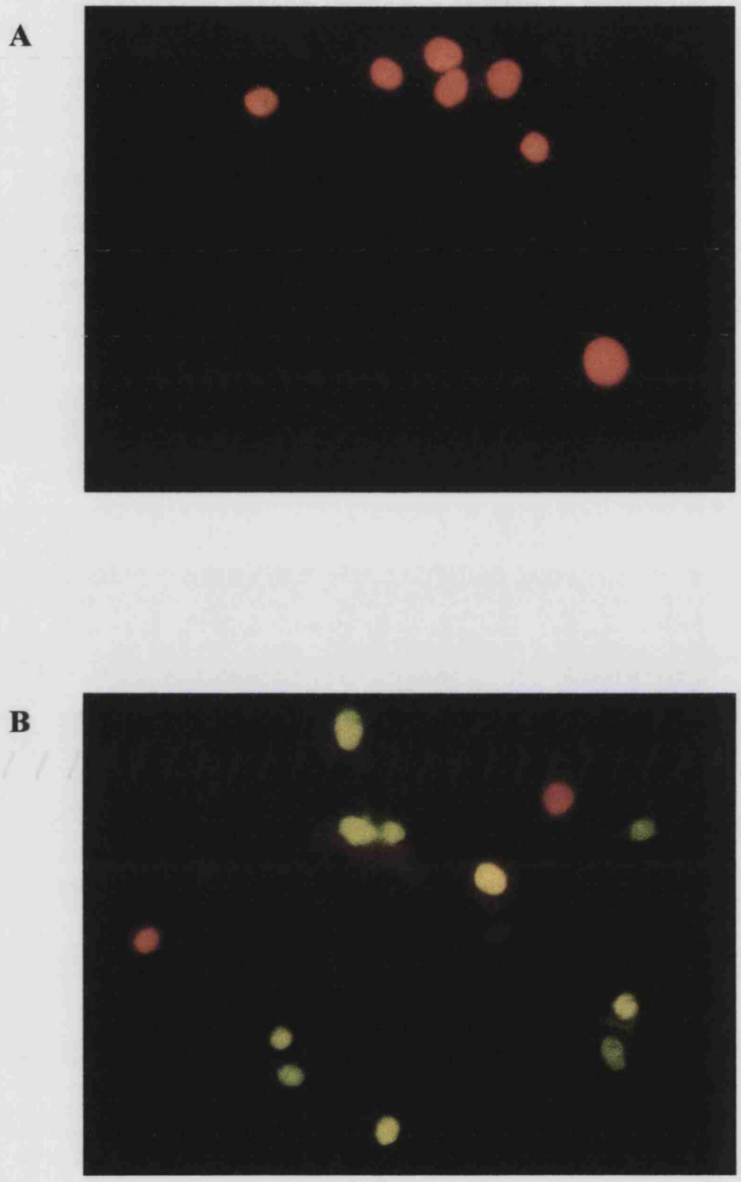
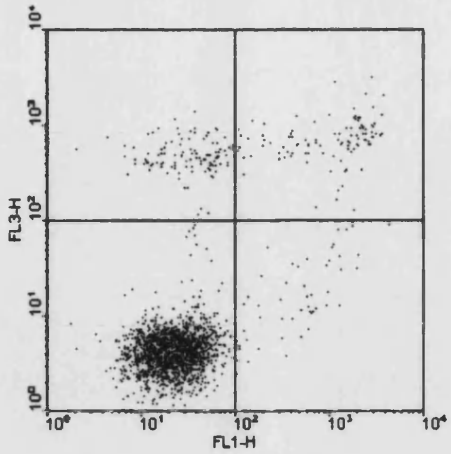


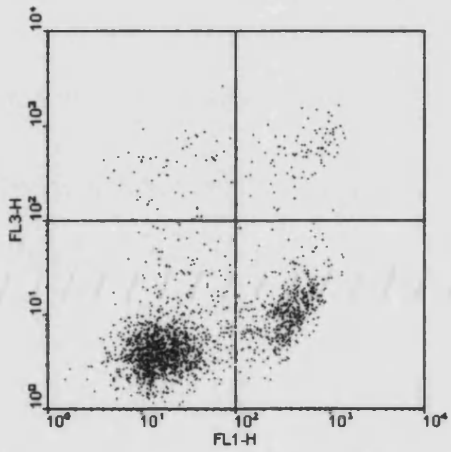
Figure 5.1A. Photographs of HT-29 cells treated as indicated for 24 hours and stained to assess DNA fragmentation (Apoptag, see section 2.2.8.1) in **A.** Vehicle and **B.** IL-1α/IFN-γ/TNF-α stimulated cells.

A



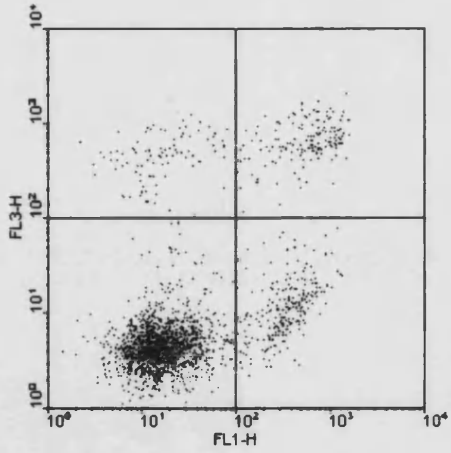
Quad	% Total
UL	7.23
UR	6.37
LL	83.02
LR	3.38

B



Quad	% Total
UL	2.46
UR	4.46
LL	67.44
LR	25.64

C



Quad	% Total
UL	4.73
UR	8.70
LL	74.52
LR	12.05

Figure 5.1B. FACS analysis of HT-29 cells stimulated as indicated for 8 hours and stained with Annexin V (LR quadrant) to assess phosphatidylserine translocation (apoptotic cells). LL indicates the % of live cells, whereas UR indicates both apoptotic and necrotic cells. **A.** Vehicle stimulated cells. **B.** IL-1α/IFN-γ/TNF-α. **C.** IL-1α/IFN-γ/TNF-α pretreated with IL-13 for 1 hour.

Having established a profile of cytokines that induce dell death in HT-29 cells, it was sought to establish whether the anti-inflammatory cytokine IL-13 played a role in the inhibition of cell death. Pre-treatment of the HT-29 cells with IL-13 (30 ng/ml) markedly inhibited apoptotic events stimulated by the pro-inflammatory cytokines (Table 5.1 and Fig. 5.1). These experiments indicate that IL-13 consistently inhibited the induction of apoptotic markers in cells treated with the combined stimuli of IL-1α/IFN-γ /TNF-α by 50-65%.

Treatment of HT-29 cells for 24 hours with IL-13 had no effect on the level of expression of TNFR1 (Fig. 5.2A) as assessed by flow cytometry using a receptor-specific antibody, thus confirming previous observations ((Manna and Aggarwal, 1998). Similarly, flow cytometry using appropriate receptor antibodies also revealed that IL-13 had no effect on the levels of expression of IL-1RI (Fig. 5.2B) or IFN-γ receptor (Fig. 5.2B), indicating that down-regulation of receptor expression cannot account for the observed reduction in cell death.

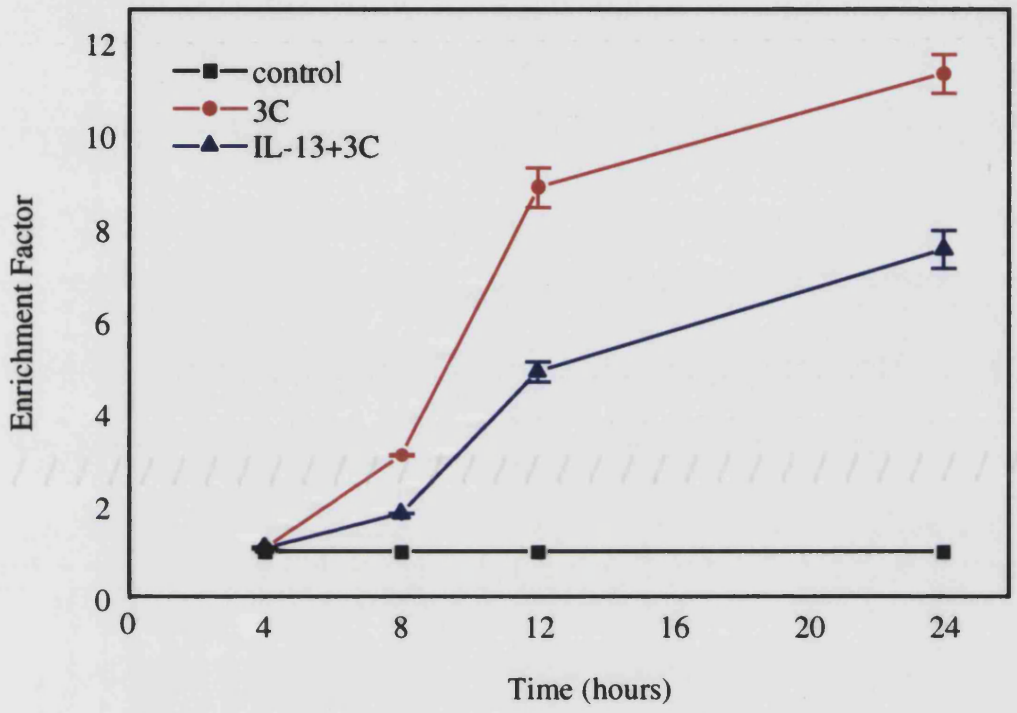
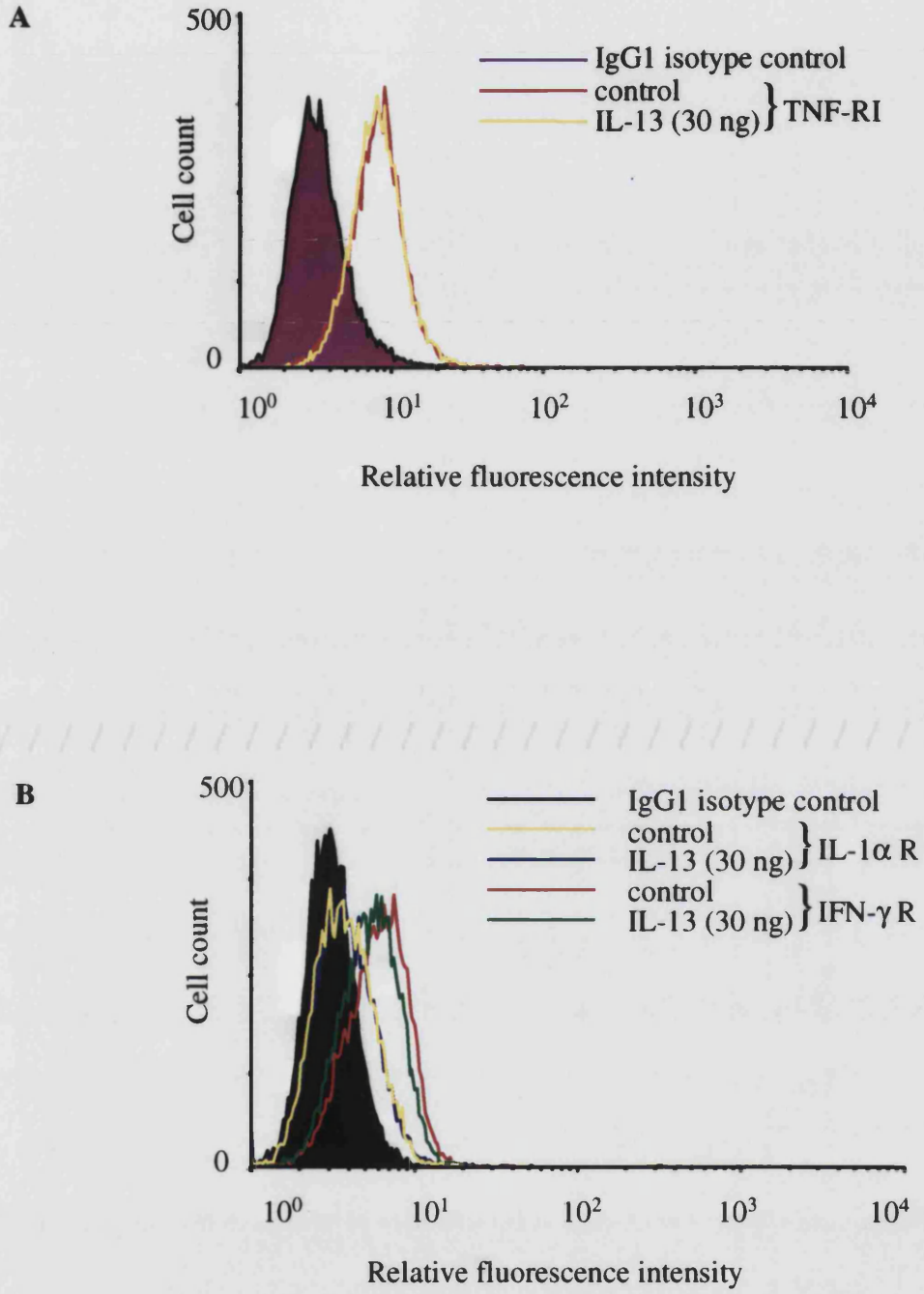


Figure 5.1C. IL-1α/IFN-γ/TNF-α-induced apoptosis in HT-29 cells is inhibited by IL-13. HT-29 cells (10^4 cells/well) were aliquoted into 96-well plates and allowed to adhere overnight. Cells were then either left untreated or treated with 30 ng/ml IL-13 for 1 hour. Where indicated, the cells were then further treated with IL-1α/IFN-γ/TNF-α or left untreated. At the times indicated, the supernatants were removed and the cell pellets were lysed and apoptosis determined by the detection of the histone-associated DNA fragments (mono- and oligonucleosomes) using the photometric cell death detection ELISA^{PLUS} assay. The data is the mean ± SEM of three separate experiments.

Table 5.1. IL-13 protects against IL-1α/IFN-γ/TNF-α-induced apoptosis of HT-29 cells. Growth arrested monolayers of HT-29 cells were either left untreated or treated with 30 ng/ml IL-13 for 1 hour. Where indicated, the cells were then treated with IL-1α (10 ng/ml), IFNγ (300 U/ml) and TNFα (100 ng/ml) or left untreated. After the times indicated, the expression of apoptotic markers by HT-29 cells was determined using either the commercial FITC-labeled annexin V binding kit or the Apoptag apoptosis detection kits as described in *Experimental Procedures.* The data is the mean ± sem of five separate experiments. **p≤0.01; *p≤0.05

	Percentage cells presenting apoptotic markers (%)			
	Annexin V		Apoptag	
Treatment (hours)	*8*	*12*	*24*	*48*
Control	3.1 ± 0.9	5.2 ± 0.9	7.1 ± 0.7	13.4 ± 1.1
IL-1α	2.8 ± 0.3	4.8 ± 0.7	7.8 ± 1.6	14.2 ± 0.8
IFN-γ	3.3 ± 0.9	5.6 ± 1.4	9.2 ± 0.8	14.7 ± 1.7
TNF-α	4.1 ± 0.8	7.8 ± 0.3	10.1 ± 1.6	16.1 ± 2.3
IL1α/IFNγ/TNFα	24.3** ± 1.2	32.3** ± 0.2	61.3** ± 1.4	81.2** ± 1.1
IL-13+IL1α/IFNγ/TNFα	11.2* ± 1.1	19.2* ± 0.2	36.7* ± 0.9	55.6* ± 2.0



5.2. IL-13 effects on cytokine receptor expression. Confluent HT-29 cells were either stimulated with vehicle or IL-13 (30 ng/ml) for 24 hours. 1 x 10^5 cells were incubated with FITC-conjugated antibodies to surface receptors. A. TNF-R1 and B. IL-1α R and IFN-γ R. Data is representative of two separate experiments.

IFN-γ is required in combination with the death-inducing factor TNF-α or CD95 ligation to induce apoptosis

Ligation of CD95 (Fas/APO-1), which is a member of the TNF receptor superfamily, is associated with the induction of apoptosis in several cell types (S. Nagata, 1997). Flow cytometry has revealed the presence of CD95 on HT-29 cells used in this study (Fig. 5.3A). We therefore performed experiments to investigate whether or not CD95 ligation stimulates apoptosis of HT-29 cells. In this respect, ligation of CD95 with the antibody CH11 did not induce apoptosis above control basal levels (Fig. 5.3B). However, treatment of HT-29 cells with IFN-γ in combination with the anti-CD95 mAb CH11, induced an approximate 9-fold increase in DNA fragmentation over 24 hours, which was comparable to the apoptosis induced by IFN-γ and TNF-α (Fig. 5.3B). By contrast, the minimum cytokine combination for the induction of iNOS and NO generation, namely IL-1α/IFN-γ, induced only a 5-fold increase in DNA fragmentation above basal levels. Preincubation with IL-13 again reduced the prevalence of apoptotic markers induced by IFNγ/CH11 and IFN-γ/TNF-α by approximately 50%, but did not inhibit apoptosis driven by IL-1α/IFN-γ (Fig. 5.3B).

Cytokine-driven iNOS activation and apoptosis are independent functional events

Several studies have demonstrated that nitric oxide regulates apoptosis in a number of settings (reviewed by Brüne *et al.*, 1998). Given that concentrations of IL-1α/IFN-γ/TNF-α that are known to induce iNOS expression and that NO production can also stimulate apoptosis, the possibility that this apoptotic response may be NO-dependent was considered. To investigate this possibility, HT-29 cells were pretreated with the iNOS

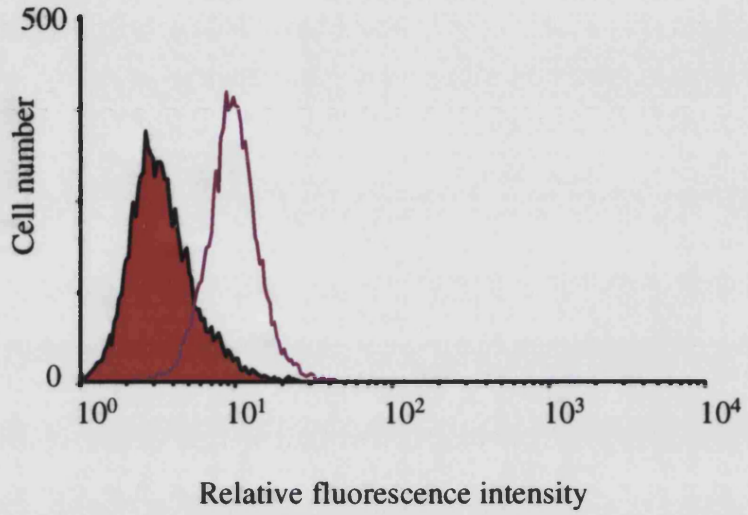
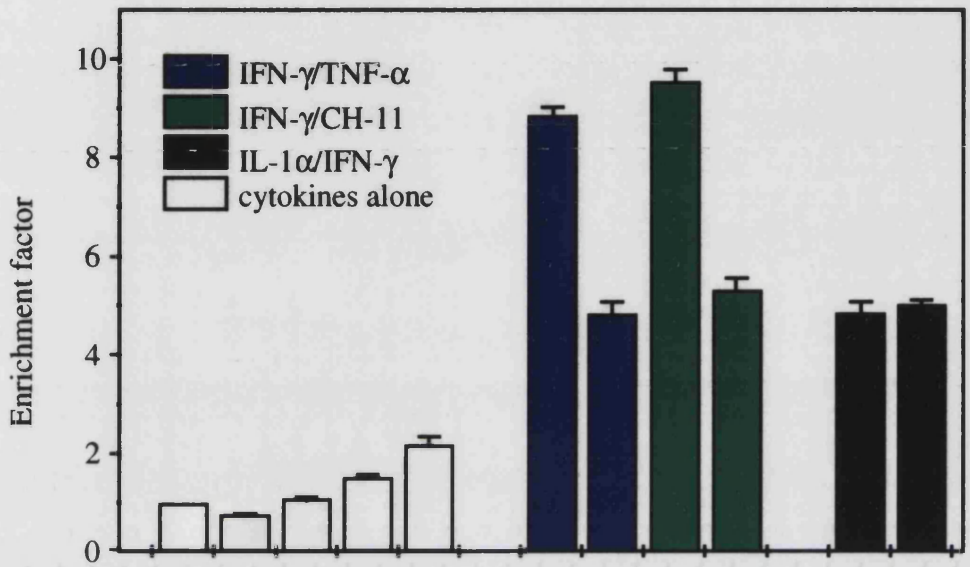


Figure 5.3A. CD95 (Fas) receptor expression in unstimulated HT-29 cells. Confluent HT-29 cells were left untreated for 24 hours. 1 x 10^5 cells were incubated with FITC-conjugated anti-CD95 as described in section 2.2.3 and analysed by FACS. The data is representative of two separate experiments.



IL-1α (10ng/ml)	+							
IFN-γ (300U/ml)		+						
TNF-α (100ng/ml)			+					
CH-11 (100ng/ml)				+				
IL-13 (30ng/ml)					+		+		+

Figure 5.3B. IFN-γ is required in combination with the death-inducing factors TNF-α or CD95 ligation to induce apoptosis. HT-29 cells were either left untreated or treated with 30 ng/ml IL-13 for 1 hour. Where indicated, the cells were then further treated with combinations of IL-1α (10 ng/ml), TNF-α (100 ng/ml), IFN-γ (300 U/ml) or CH11 (100 ng/ml) or left untreated. After 24 hours, the supernatants were removed and the cell pellets were lysed. Apoptosis was determined using the photometric cell death detection ELISA^{PLUS} assay.The data is the mean ± SEM of three separate experiments.

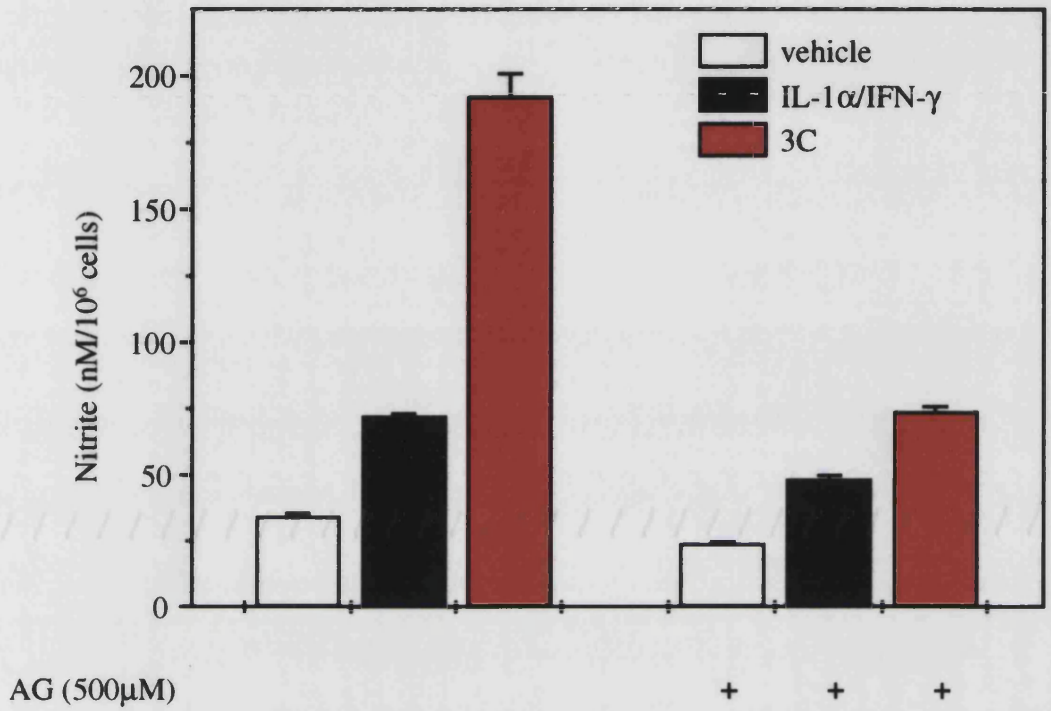


Figure 5.4A. Effect of iNOS inhibitor on cytokine-induced nitrite production. HT-29 cells (3.5 x 10^6 cells/well) were treated for 10 minutes with vehicle or with 500 μM aminoguanadine (AG) as indicated at 37°C. The cells were then further treated with combinations of IL-1α (10 ng/ml), TNF-α (100 ng/ml) or IFN-γ (300 U/ml) as indicated or left untreated. Nitrite production in the supernatants was measured after 24 hours. The data is the mean ± SEM of three separate experiments.

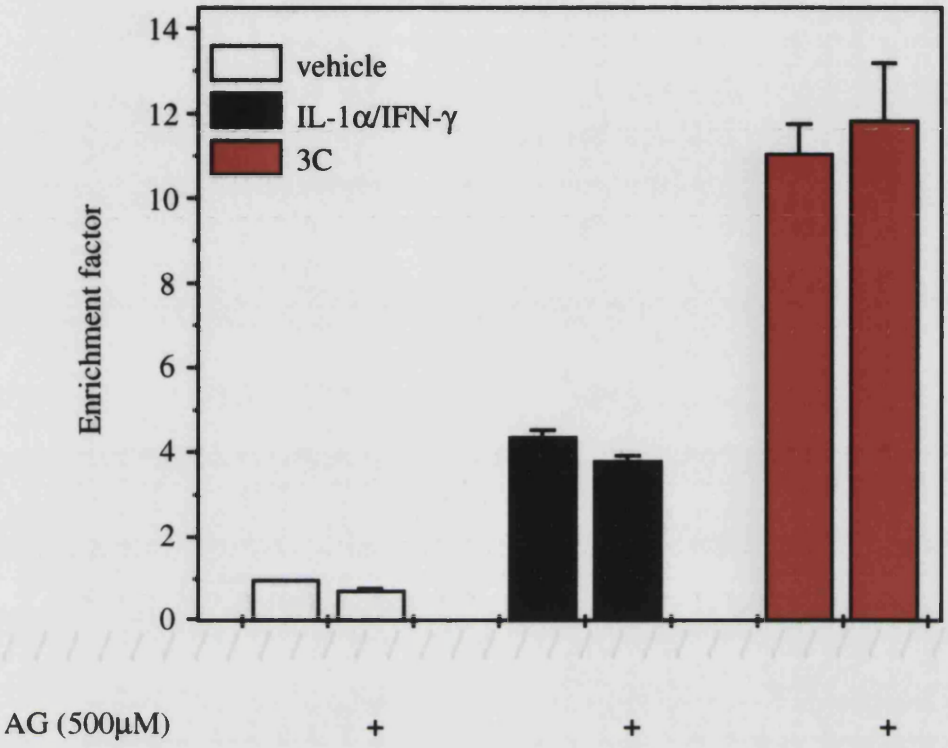


Figure 5.4B. Effect of iNOS inhibitor on cytokine-induced apoptosis. HT-29 cells were treated for 10 minutes with vehicle or with 500 μM aminoguanadine (AG) as indicated and then treated with combinations of IL-1α (10 ng/ml) TNF-α (100 ng/ml) and IFN-γ (300 U/ml) as indicated or left untreated for 24 hours. Supernatants were removed and the cell pellets were lysed. Apoptosis was determined by using the photometric cell death detection ELISA^{PLUS} assay. The data is the mean ± SEM of three separate experiments.

inhibitor 500μM aminoguanidine, which markedly inhibited the concentration of nitrite generated by IL-1α/IFN-γ/TNF-α over 24 hours (Fig. 5.4A). Less inhibition was observed with the minimum combination of IL-1α/IFN-γ. Aminoguanidine had no effect on the apoptotic signals provided by combinations of either IL-1α/IFN-γ/TNF-α or IL-1α/IFN-γ (Fig. 5.4B).

Initial apoptotic events are known to result in the activation of the proteolytic enzyme cascades involving caspases, which cleave specific proteins and irreversibly commit the cell to apoptotic death (Salvesen and Dixit, 1997). Inhibition of this proteolytic cascade can be achieved using a broadly selective caspase inhibitor Z-VAD-FMK (Villa *et al.*, 1997). To assess whether cytokine-driven iNOS production was dependent upon activation of caspases, cells were pre-treated with 50 μM Z-VAD-FMK, which completely abrogated the apoptosis of HT-29 cells stimulated by IL-1α/IFN-γ/TNF-α, IL-1α/IFN-γ, IFN-γ/TNF-α and IFN-γ/CH11 (Fig. 5.5A). However, the caspase inhibitor did not interfere with the observed ability of HT-29 cells to generate nitrite in response to IL-1α/IFN-γ/TNF-α or IL-1α/IFN-γ (Fig. 5.5B), as does pretreatment with IL-13. Furthermore, IFN-γ/TNF-α and IFN-γ/CH11 are not able to induce iNOS or stimulate the generation of nitrite, but are able to stimulate apoptosis (Fig. 5.5A). Together, these data indicate that cytokine driven iNOS activation and apoptosis are separable, independent events.

Reversal of IL-13-induced inhibition of apoptosis by wortmannin and LY 294002

As previously shown, IL-13 strongly activates the lipid kinase PI 3-kinase (Wright *et al.*, 1997). Activation of this pathway is believed to be a pivotal upstream component of a signalling cascade important in promoting cell survival events in many cell types (Kauffman-

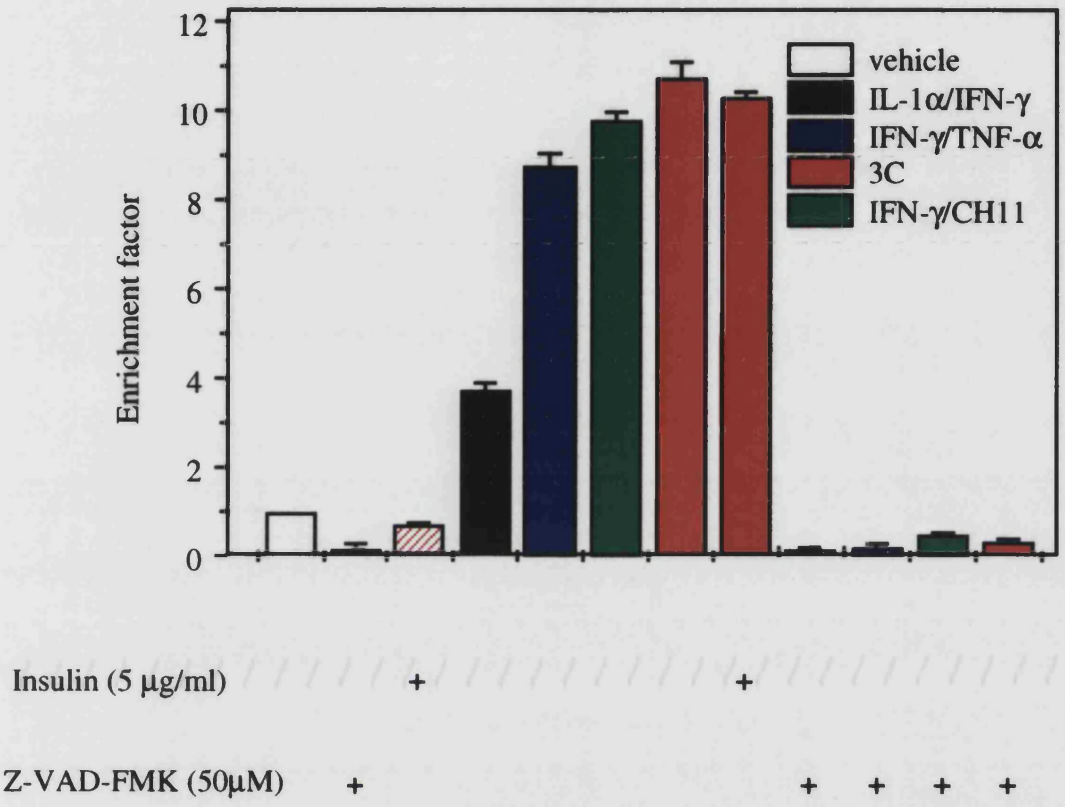


Figure 5.5A. Effect of the caspase inhibitor Z-VAD-FMK on cytokine-induced apoptosis. HT-29 cells were either left untreated or treated with 50 µM Z-VAD-FMK or 5 µg/ml insulin for 1 hour . Where indicated, the cells were then further treated with combinations of IL-1α (10 ng/ml), TNF-α (100 ng/ml), IFN-γ (300 U/ml) or CH11 (100 ng/ml) or left untreated. After 24 hours, the supernatants were removed and the cell pellets were lysed. Apoptosis was determined by using the photometric cell death detection ELISA^{PLUS} assay. The data is the mean ± SEM of three separate experiments.

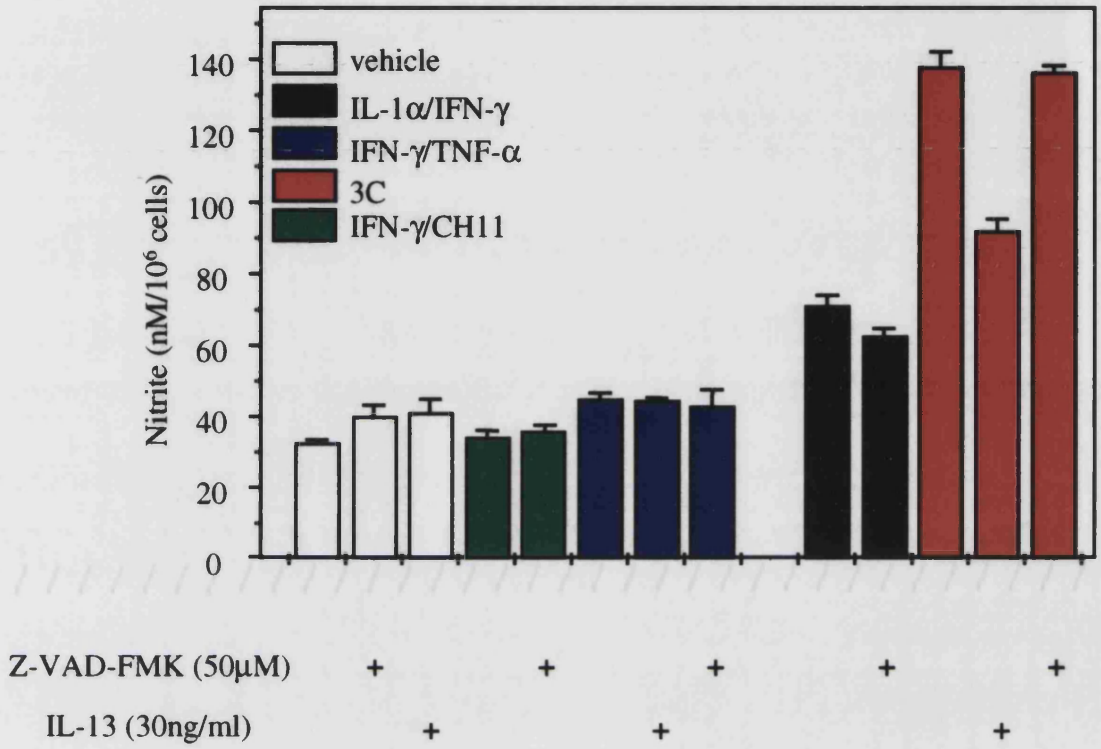
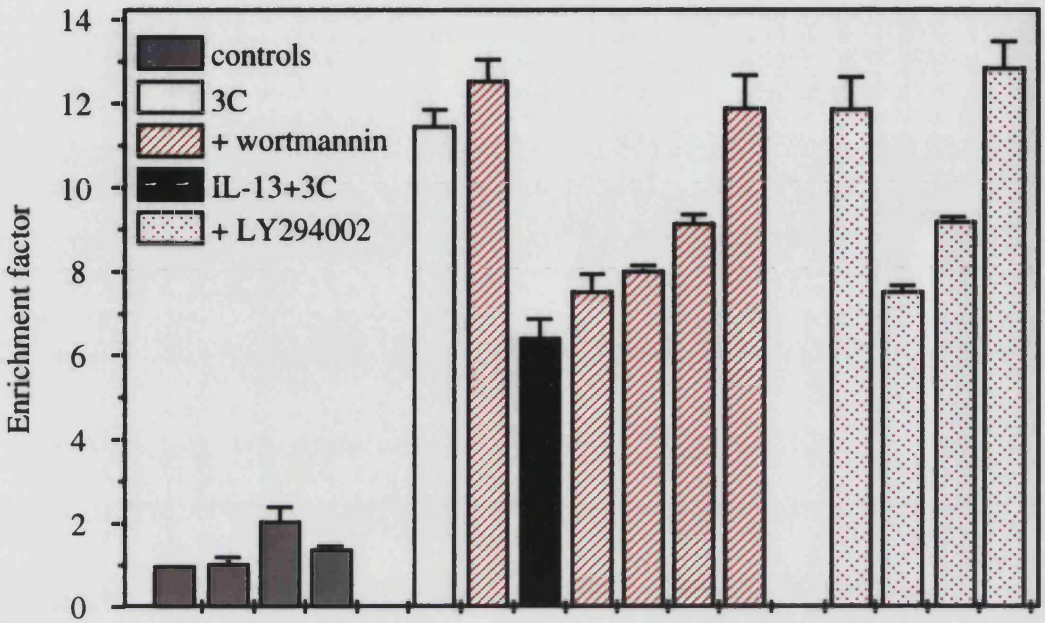


Figure 5.5B. Effect of the caspase inhibitor Z-VAD-FMK on cytokine-induced nitrite production. HT-29 cells (3.5 x 10^6 cells/well) were treated for 1 hour with vehicle or with 50 μM Z-VAD-FMK as indicated. The cells were then further treated with combinations of IL-1α (10 ng/ml), TNF-α (100 ng/ml) or IFN-γ (300 U/ml) as indicated or left untreated. Nitrite production in the supernatants after 24 hours was measured. The data is the mean ± SEM of three separate experiments.



IL-1α/IFN-γ/TNF-α				+	+	+	+	+	+	+		+	+	+	+
Wortmannin (nM)		100			100		10	30	100	300					
IL-13 (30ng/ml)	+					+	+	+	+	+			+	+	+
LY294002 (μM)			10									10	1	10	30

Figure 5.6A. PI 3-kinase inhibitors prevent IL-13 suppression of cytokine-induced apoptosis of HT-29 cells. HT-29 cells were treated for 10 minutes with vehicle, wortmannin or LY294002 as indicated. Cells were then either left untreated or treated with 30 ng/ml IL-13 for 1 hour. Where indicated, cells were then further treated with IL-1α/IFN-γ/TNF-α or left untreated. After 24 hours, the supernatants were removed and the cell pellets were lysed. Apoptosis was determined by the ELISA^{PLUS} assay. The data is the mean ± SEM of three separate experiments.

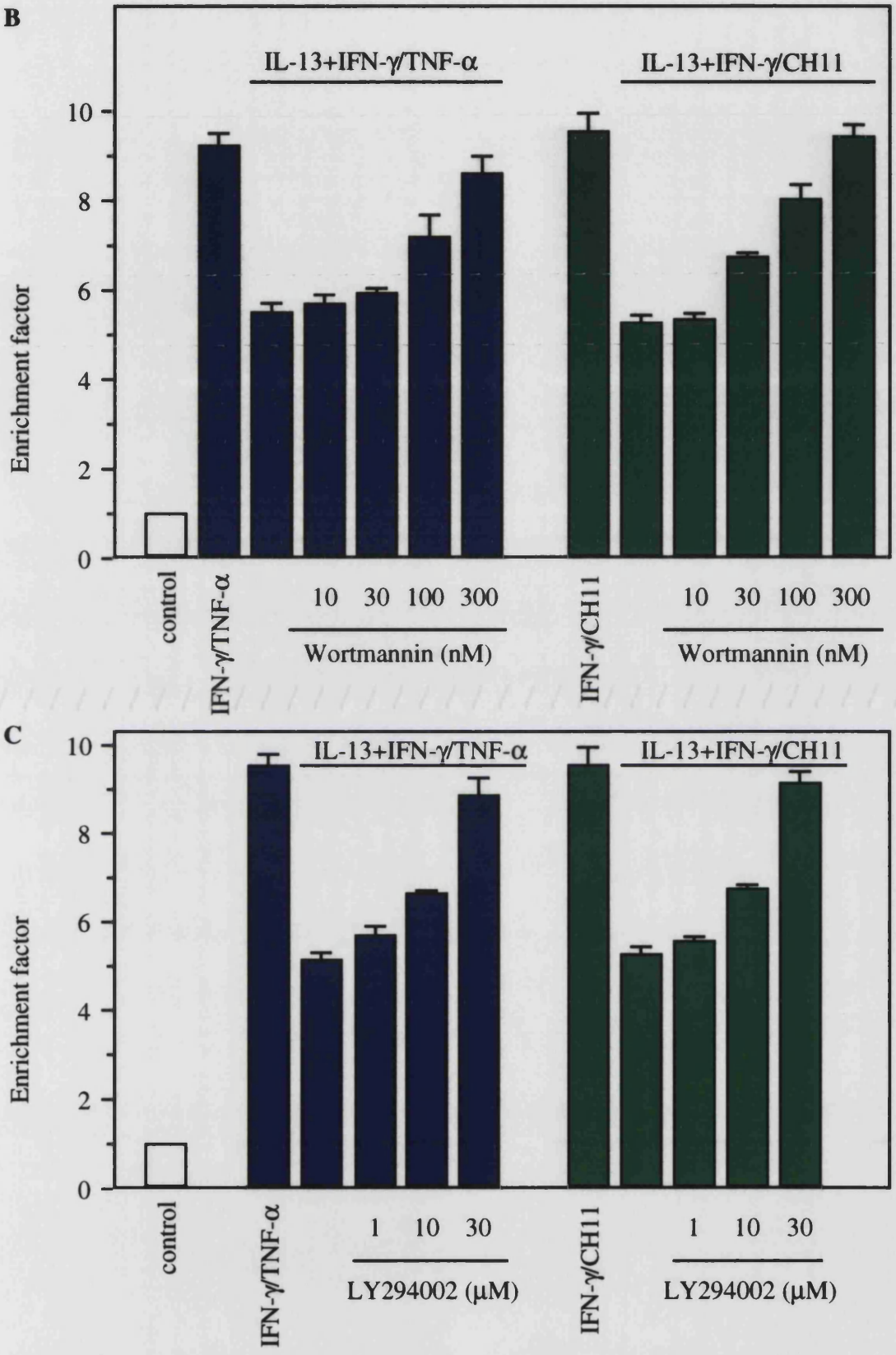


Figure 5.6. PI 3-kinase inhibitors prevent IL-13 suppression of cytokine-induced apoptosis of HT-29 cells. HT-29 cells were treated for A. 10 minutes with vehicle or wortmannin (nM) or B. LY294002 (μM) for 15 minutes, as indicated. Cells were then either left untreated or treated with 30 ng/ml IL-13 for 1 hour. Where indicated, the cells were then further treated with IFN-γ/TNF-α or IFN-γ/CH-11 or left untreated. After 24 hours, apoptosis was determined by the ELISA^{PLUS} assay. The data is the mean ± SEM of three separate experiments.

Zeh *et al.*, 1997; Dudek *et al.*, 1997; Kulik *et al.*, 1997 and Yao and Cooper, 1995). Hence, to investigate the role of PI 3-kinase in mediating the anti-apoptotic effects of IL-13 on IL-1α/IFN-γ/TNF-α-stimulated apoptosis of HT-29 cells, the PI 3-kinase inhibitors wortmannin and LY294002 were used. Pre-incubations of wortmannin (10-300 nM) for 10 minutes before cytokine treatments were able to dose dependently reverse the ability of IL-13 to protect HT-29 cells from cytokine-induced apoptosis (Fig. 5.6A). Equally, the structurally unrelated PI 3-kinase inhibitor, LY294002 (1-30 μM), was also able to reverse the IL-13 effect (Fig. 5.6A). Similarly, treatment of HT-29 cells with either wortmannin (Fig. 5.6B) or LY294002 (Fig. 5.6C), also prevented the IL-13-mediated inhibition of apoptosis induced by either IFN-γ/TNF-α or IFN-γ/CH-11.

IL-13 activates the PI 3-kinase effector protein kinase B

PKB is a major downstream effector of the PI 3-kinase-dependent signalling cascade and has been shown to be a key mediator required for growth factor-induced cell survival and protection against c-Myc-induced cell death in fibroblasts (Kauffman-Zeh *et al.*, 1997; Dudek *et al.*, 1997 and Kulik *et al.*, 1997). We therefore investigated whether the protective affects of IL-13 on IL-1α/IFN-γ /TNF-α-stimulated apoptosis in HT-29 cells, correlated with IL-13 activation of PKB. Hence, cell lysates derived from resting and IL-13 stimulated cells were immunoblotted using a phosphospecific antibody to the phosphorylated active form of PKB. IL-13 can be shown to activate PKB within 5 minutes stimulation, up to a maximum at 10 minutes, and is comparable in magnitude with PKB activation observed in response to insulin (5μg/ml) treatment as a positive control (Fig. 5.7A). Insulin however, provided only a 10-15% protection against cytokine-induced apoptosis (Fig. 5.5A). The IL-13-stimulated activation of PKB appears to be sustained for

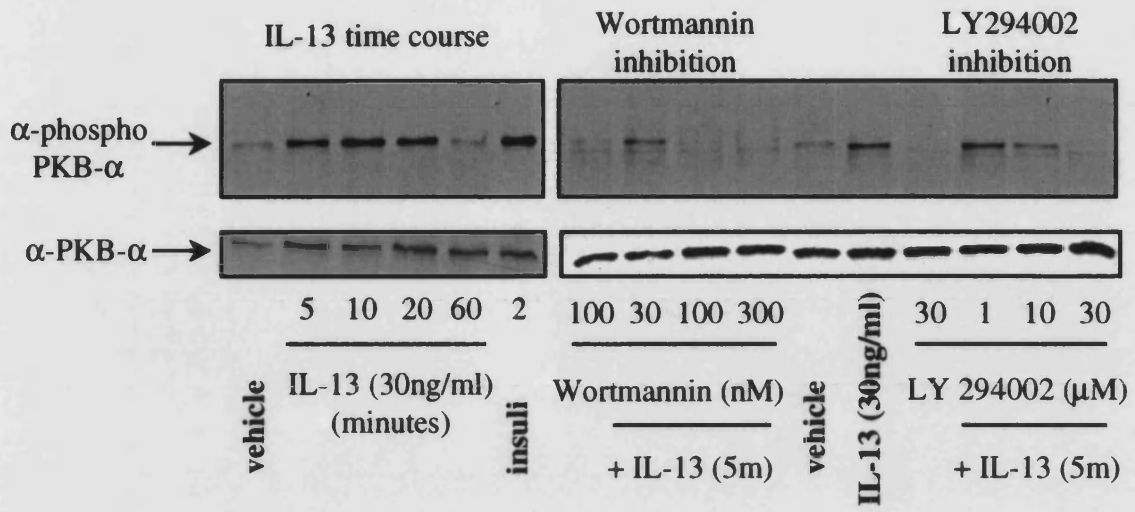


Figure 5.7A. HT-29 cells (10^7) were treated for 10 minutes with vehicle (*left panel*), 30-300 nM wortmannin (*right panel*) or 1-30 μM LY294002 (*right panel*) as indicated at 37°C. Cells were then left unstimulated or further treated with 30 ng/ml IL-13 or 5 μg/ml insulin (as a positive control) at 37°C for the times indicated. The HT-29 cells were lysed and the lysates were immunoblotted with a phosphospecific PKB antibody with affinity for the ^{473}Ser-phosphorylated, active form of PKB. Data is from a single experiment representative of at least three others.

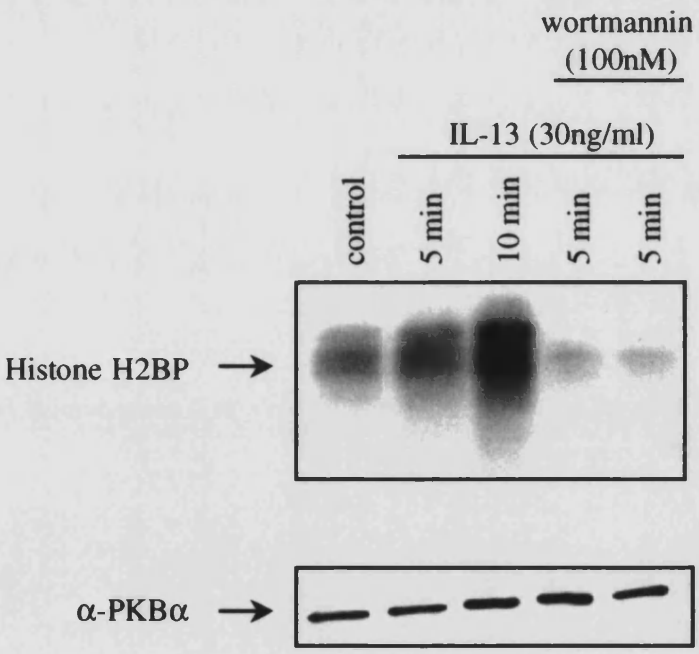


Figure 5.7B. 10^7 HT-29 cells were stimulated with 30 ng/ml IL-13 for the times indicated with or without a 10 minute pre-incubation of 100 nM wortmannin. After cell lysis and immunoprecipitation with α-PKB, immunocomplexe were subjected to an *in vitro* kinase assay as described in section 2.2.9.2. Data is from a single experiment representative of at least three others.

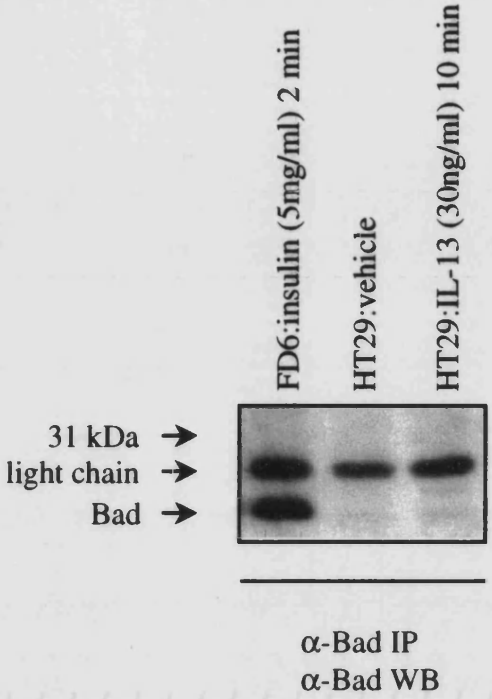


Figure 5.8. Effect of IL-13 on Bad hyperphosphorylation in HT-29 cells. 10^7 HT-29 cells were either left untreated or treated with 30 ng/ml IL-13 for the times indicated. Cells were lysed and the lysates were subjected to immunoprecipitation with an anti-Bad mAb. The immunoprecipitates were washed and proteins separated by SDS-PAGE using a low-bis gel, transferred to nitrocellulose and immunoblotted for Bad expression. FD6 cells stimulated with insulin were used as a positive control for Bad expression. Representative data of at least three experiments.

up to 20 minutes, but has returned to control levels after 1 hour. Both wortmannin and LY294002 were able to inhibit this signal, adding further evidence that the activation of PI 3-kinase by IL-13 leads to the activation of PKB and that this pathway is anti-apoptotic in this system. Blots were stripped and reprobed with an anti-PKB antibody provided in the kit to verify equal loading and efficacy of protein transfer (Fig. 5.7A). In addition, endogenous PKB was immunoprecipitated from IL-13-stimulated cells in the presence and absence of the PI 3-kinase inhibitor, wortmannin, and the immunoprecipitates were assayed for *in vitro* PKB activity. This approach confirmed that PKBα was activated by IL-13 in a PI 3-kinase-dependent manner (Fig. 5.7B).

The mechanism by which PKB is believed to promote cell survival involves the serine phosphorylation of the death promoting Bcl-2 family member, Bad. This results in the dissociation of Bad from Bcl-x_L, thus allowing Bcl-x_L to act as a survival factor (Datta *et al.*, 1997; del Peso *et al.*, 1997 and J. Downward, 1999). Immunoblotting of HT-29 whole cell extracts revealed no detectable amounts of Bad. However, immunoprecipitation and immunoblotting of Bad, after electrophoresis through a low-bis, 12.5% acrylamide gel, revealed a barely detectable hyperphosphorylated form of Bad as characterized by its shift in gel mobility (Fig. 5.8).

SH_2-containing inositol 5-phosphatase induced to bind to an unknown protein by IL-13

SHIP hydrolyses the signalling molecule, PI (3,4,5) P_3 and has a negative function by terminating signals (Majerus *et al.*, 1999). There was the possibility that activation of SHIP in HT-29 cells may serve as a negative regulator of IL-13-induced PI 3-kinase activation. Immunoprecipitation of stimulated HT-29 cells with a peptide construct of the SH_2 domain

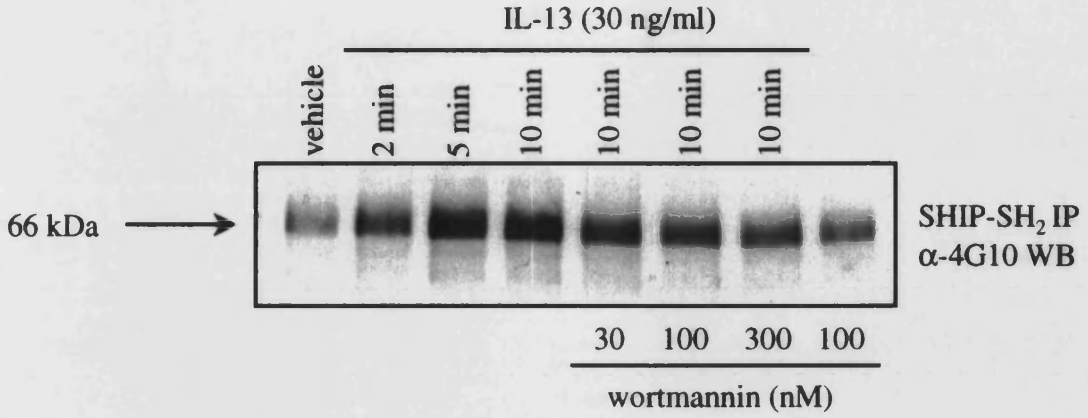


Figure 5.9. HT-29 cells (10^7) were stimulated with vehicle or wortmannin (100nM) for 10 minutes prior to either vehicle or IL-13 (30 ng/ml) for the times shown. Lysates were subjected to immunoprecipitation with an antibody raised against the SH_2 domain of SHIP and Western analysis. This is an anti-phosphosphotyrosine blot showing the tyrosine phosphorylation of a broad band between 65-70 kDa. Data is representative of two separate experiments. Membranes generated from both experiments were stripped and reprobed for Shc and SHPTP2 with negative results.

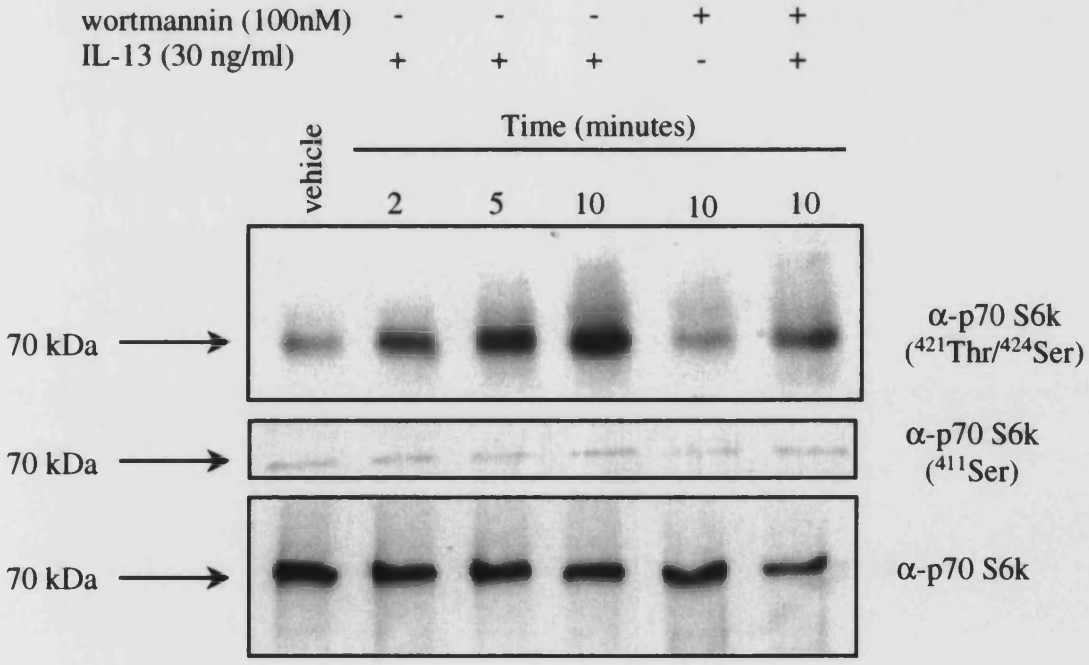


Figure 5.10 HT-29 cells (10^7) were treated with vehicle or wortmannin (100nM) for 10 minutes prior to the addition of vehicle or IL-13 (30 ng/ml) for the times indicated. Whole cell extracts were subjected to SDS-PAGE and Western analysis. Two phosphorylated forms of p70 S6 kinase, as indicated, were used to probe for activity of this kinase post-IL-13 stimulation. In addition, membranes were stripped and reprobed with pan-p70 S6 kinase (bottom panel) to verify equal loading. n=2.

of SHIP, conjugated to the GST fusion protein (a kind donation by Heather Bone), was performed and subjected to Western analysis with the anti-phosphotyrosine antobody, 4G10 (Figure 5.9). An unknown protein, of approximately 65 – 70 kDa, was basally tyrosine phosphorylated and this activity increased over 10 minutes. Wortmannin was able to inhibit this activity in a concentration-dependent manner. This would suggest a direct interaction between this protein and the SH_2 domain of SHIP, an interaction that might be PI 3-kinase-dependent. In an attempt to identify the unknown protein, blots were stripped and reprobed for the adapter protein Shc and the phosphotyrosine phosphatases SHP1 and SHP2 (all of which fall within the kDa range of the unknown protein) with negative results.

IL-13 induces the phosphorylation of p70 S6-kinase

The translational control of mRNA transcripts containing 5' polypyrimidine tracts involves p70 S6-kinase and the complex regulation of this enzyme involves phosphorylation at many sites (C. G. Proud, 1996). As p70 S6-kinase is a downstream effector of PI 3-kinase (Weng *et al.*, 1995 and Chung *et al.*, 1994), resting and stimulated HT-29 whole cell extracts were immunoblotted using phosphospecific antibodies to two phosphorylated forms of p70 S6-kinase (Fig. 5.10). IL-13 induced an increase in phosphorylation at the ^{421}Thr/^{424}Ser sites over 10 minutes, but not at the ^{411}Ser site. Wortmannin was able to inhibit the IL-13-induced phosphorylation, although not to basal levels. Blots were also stripped and reprobed with an anti-p70 S6-kinase antibody to verify equal loading and efficient transfer.

IL-13 delays cytokine-induced activation of the caspase cascade

Activation of the caspase cascade is pivotal to the death execution phase of apoptosis and it appears that caspase-8 is the apical member of the pathway induced by CD95 and TNFR-1 with caspase-3 lying downstream (Boldin *et al.*, 1996; Muzio *et al.*, 1996 and Enari *et al.*, 1996). Hence, we investigated whether the induction of apoptotic markers induced by various cytokine combinations also correlated with activation of caspase-8 and -3. Indeed, treatment of cells with IFN-γ/TNFα and IL-1α/IFN-γ/TNF-α, but not IL-1α/IFN-γ, stimulated both caspase-8 and caspase-3 activity (Fig. 5.11A). In comparison, while treatment with IL-1α did not stimulate caspase activity, both TNF-α and IFN-γ elicited modest activation of these caspases (Fig. 5.11A). Interestingly, pre-treatment of HT-29 cells with IL-13 consistently induced a partial inhibition of IL-1α/IFN-γ/TNF-α-stimulated caspase-8 and caspase-3 activity at early time points (e.g., 12 hours) which may constitute a delay in caspase activation by IL-13 (Figs. 5.11B and C).

5.2 DISCUSSION

In this report it has been demonstrated that a combination of pro-inflammatory cytokines, namely IL-1α/IFN-γ/TNF-α, stimulates the expression of apoptotic markers in approximately 25 - 80% of cytokine-treated HT-29 cells (depending on time of analysis) as evidenced by assays that detect DNA fragmentation and externalisation of phosphatidylserine. Apoptosis can also be stimulated to varying extents by the combination of IL-1α/IFN-γ or IFN-γ /TNF-α. Moreover, we present evidence that the induction of these apoptotic markers is not dependent on the expression of iNOS and NO production.

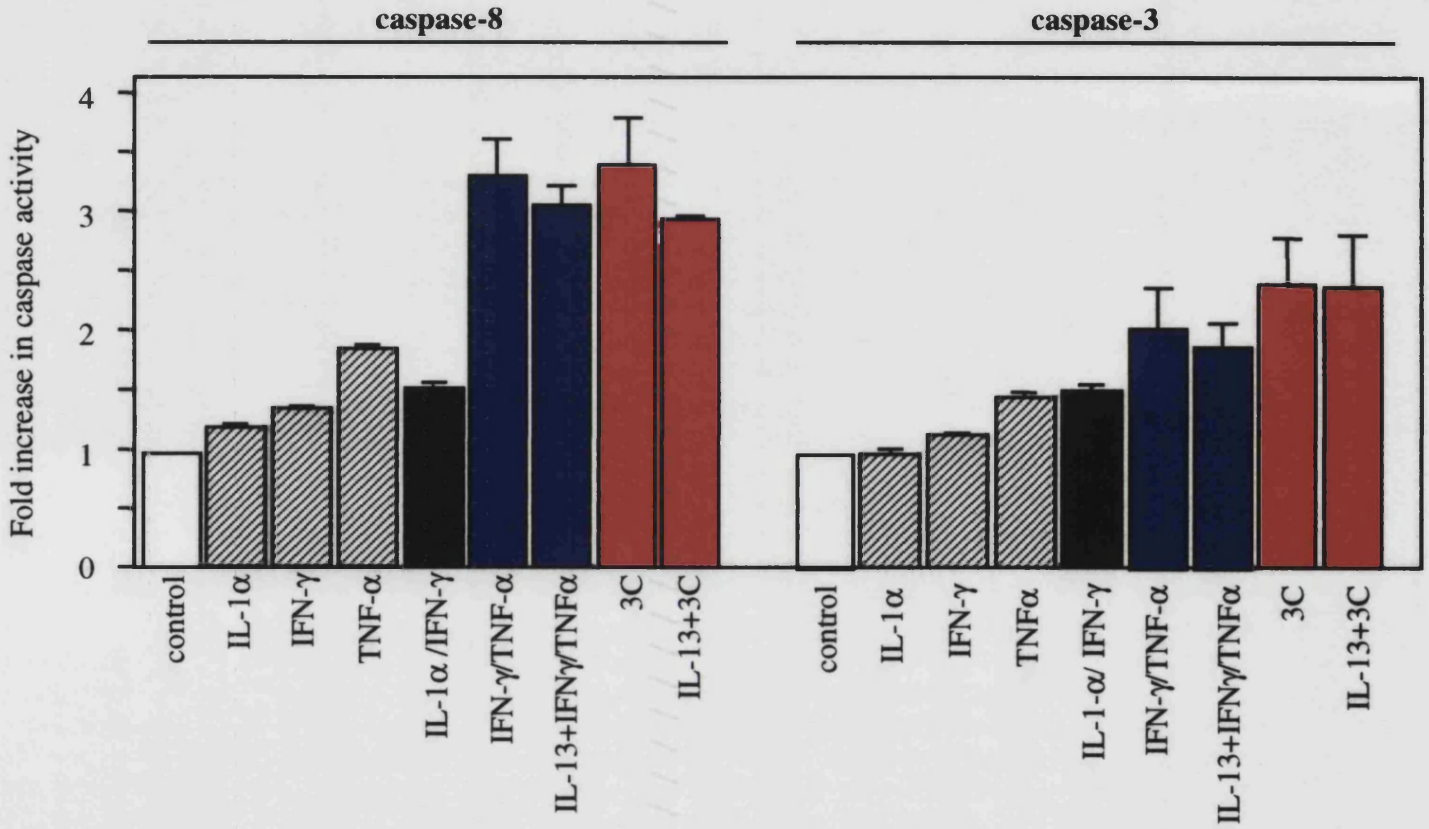


Figure 5.11A. Effect of IL-13 on cytokine-induced activation of caspase-8 and caspase-3 in HT-29 cells. HT-29 cells were either left untreated or treated with 30 ng/ml IL-13 for 1 hour. Where indicated, the cells were then further treated with combinations of cytokines as indicated or left untreated. After 24 hours, cells were pelleted to include any floating cells, the supernatants were removed and the cell pellets were lysed and assayed for caspase-8 and caspase-3 activity. Data is the mean of triplicates from one experiment ± SEM, representative of two independent experiments.

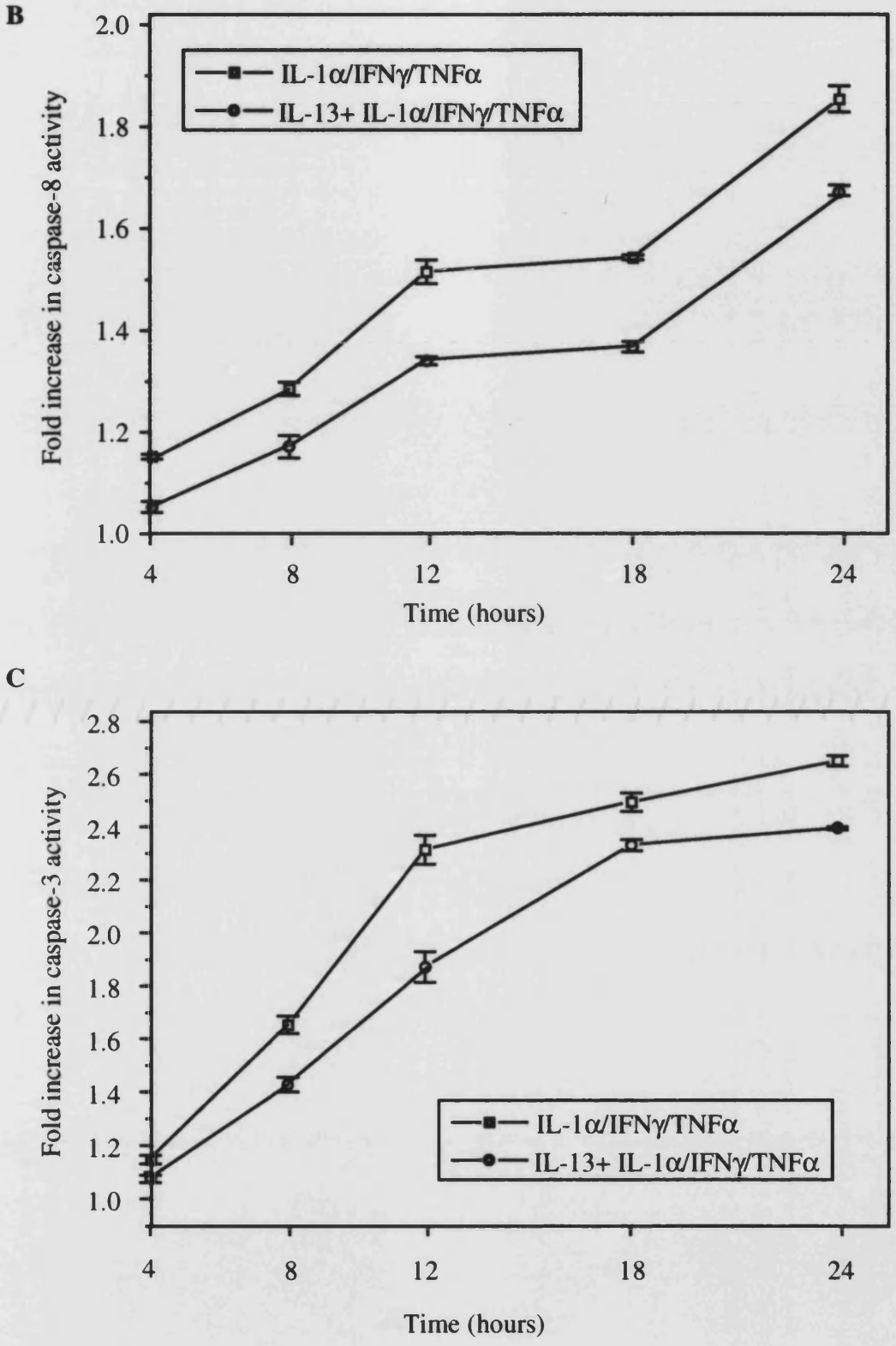


Figure 5.11. HT-29 cells (3.5 x 10^6) were either left untreated or treated with IL-13 (30 ng/ml) for 1 hour. After 24 hours, cells were pelleted to include any floating cells, the supernatants removed and the cell lysed and assayed for B. caspase-8 and C. caspase-3 activity, as described in section 2.2.8.4. Data are the mean of triplicate from one experiment ± SEM, representative of three independent experiments.

Furthermore, pre-treatment with the anti-inflammatory cytokine IL-13 protects against IL-1α/IFN-γ/TNF-α-, IFN-γ/TNF-α- and IFN-γ/CH11-induced (but not IL-1α/IFN-γ-induced) cell death in this system via a PI 3-kinase-dependent mechanism. IL-13 is also known to prevent induction of NO production by HT-29 cells in response to IL-1α/IFN-γ/TNF-α (Kolios *et al.*, 1995 and Wright *et al.*, 1997). This correlates with the first demonstration that IL-13 stimulates activation of the major downstream PI 3-kinase effector PKB, a signalling pathway thought to mediate the promotion of cell survival in a number of cell systems (Kauffman-Zeh *et al.*, 1997; Dudek *et al.*, 1997 and Kulik *et al.*, 1997). However, IL-13-induced phosphorylation of the downstream target of PKB, Bad, was barely detectable, suggesting that this is an unlikely target for PKB activity in this system. In addition, IL-13 pre-treatment partially delayed, but did not did not prevent cytokine-stimulated activation of either caspase-8 or caspase-3.

Individually, IL-1α, IFN-γ and TNF-α do not induce apoptosis of HT-29 cells. However, our observation that combinations of IL-1α, IFN-γ and TNF-α induce apoptosis of HT-29 cells, correlates well with recent reports that IFN-γ increases the sensitivity of HT-29 cells to pro-apoptotic agents such as TNF-α by directly and indirectly inducing select apoptosis-related genes (Ossina *et al.*, 1997). In addition, it has been shown previously that this combination of cytokines stimulates NO production from HT-29 cells and there is considerable evidence that NO can promote apoptosis in other systems (Brüne *et al.*, 1998). Indeed, iNOS transcripts can be detected 6 hours after cytokine treatment (Kolios *et al.*, 1995) and this appears to precede cell death, which is detectable at 8 hours post-cytokine stimulation. However, there are several lines of evidence to indicate that cytokine-driven iNOS and apoptosis are independent functional events. First, the iNOS inhibitor aminoguanadine prevented IL-1α/IFN-γ /TNF-α and IL-1α/IFN-γ -induced NO production,

but had no effect on the apoptosis stimulated by these combinations of cytokines. Second, apoptosis can also be stimulated by IFN-γ/TNF-α and IFN-γ/CH11 which are unable to stimulate NO production. Third, inhibition of IL-1α/IFN-γ/TNF-α-induced apoptosis by Z-VAD-FMK had no effect on NO production induced by these cytokines. Although markers of cytokine-stimulated apoptosis, such as DNA fragmentation and phosphatidylserine externalisation, are detectable from 8-24 hours, time course experiments have revealed that other functional responses continue unabated. For instance, identical cytokine treatment can also stimulate up-regulation of iNOS and chemokine mRNA up to 24 hours post-stimulation (Kolios *et al.*, 1995 and 1996) and these responses can be down-regulated by pre-treatment with IL-13 (Kolios *et al.*, 1996 and 1999 and Wright *et al.*, 1997). So, cytokine-induced expression of apoptotic markers and events does not necessarily correlate with abrogated cell function, at least in the time frame studied here.

Activation of the proteolytic cascade by caspases appears to be essential to cytokine-induced apoptosis of HT-29 cells, given our observation that pre-treatment with the caspase inhibitor Z-VAD-FMK completely prevents apoptosis induced by IL-1α/IFN-γ/TNF-α, IL-1α/IFN-γ, IFN-γ/TNF-α and IFN-γ/CH11. This is particularly interesting given that both IL-1α and TNF-α (Schutze *et al.*, 1994) and CD95 (Cifone *et al.*, 1994) ligation have been reported to activate the ceramide pathway, which has also been implicated as a signalling pathway involved in apoptosis (Obeid *et al.*, 1993 and Pushkareva *et al.*, 1995). However, since the apoptosis of HT-29 cells stimulated by these cytokine combinations is completely inhibited by the caspase inhibitor Z-VAD-FMK, this may indicate that ceramide production is not sufficient for cell death in this system. Indeed, it has recently been shown that IFN-γ was unable to induce changes in sphingolipid levels in HT-29 cells (Veldman *et al.*, 1998), suggesting that ceramide-mediated signalling pathways may be cell-type specific. It is also

interesting to note that while treatment of HT-29 cells with TNF-α or IFN-γ resulted in modest stimulation of caspases –8 and –3, this is insufficient to drive cell death, since neither TNF-α nor IFN-γ alone stimulated apoptosis in this system. This is in marked contrast to the TNF-α-induced apoptosis observed in neutrophils and T lymphocytes which correlates well with caspase activation (Aggarwal *et al.*, 1999 and Yamashita *et al.*, 1999).

Even though IL-13 exerts a protective effect against cell death induced by IL-1α/IFN-γ/TNF-α, IFN-γ/TNF-α and IFN-γ/CH11, IL-13 pre-treatment was unable to completely inhibit cytokine-activated caspase-8 and caspase-3. Rather, it appears that IL-13 delays activation of these caspases by IL-1α/IFN-γ/TNF-α and in this respect it is interesting to note that IL-13 provides only a partial protection against cell death induced by IL-1α/IFN-γ/TNF-α, IFN-γ/TNF-α and IFN-γ/CH11. Caspase activation is required for the execution of cell death in an apoptotic manner, reviewed in (Salvesen and Dixit, 1997), but the order of caspase activation cascades is not absolute and the commitment to live or die may originate from the mitochondria, reviewed in (Green and Kroemer, 1998). Hence, while IL-13 partially inhibits and possibly delays activation of caspases –8 and –3, there may well be additional targets of IL-13-activated biochemical signals that mediate cell survival at some point distal to the apical caspase-8 and the downstream caspase-3, possibly involving mitochondrial activity. It is certainly possible that the cytokine combinations used in this study activate other upstream and downstream caspases. Indeed, it has recently been shown that the kinase Akt can phosphorylate caspase-9 and inhibit its protease activity (Cardone *et al.*, 1998). This would fit nicely with these observations, in that IL-13 can provide only partial protection against cell death induced by IL-1α/IFN-γ/TNF-α, IFN-γ/TNF-α, whereas apoptosis stimulated by IL-1α/IFN-γ was unaffected by IL-13. Hence, it appears

that multiple death promoting pathways with different sensitivity to IL-13-activated cell survival mechanisms, are activated by the cytokine combinations used in this study.

The protective effects of IL-13 against IL-1α/IFN-γ/TNF-α-, IFN-γ/TNF-α- and IFN-γ/CH11-induced apoptosis are dependent on the PI 3-kinase dependent signalling pathway, since the PI 3-kinase inhibitors wortmannin and LY29002 abrogated the protective effects of IL-13. These observations are consistent with demonstrations that the PI 3-kinase dependent signalling pathway and, in particular, its downstream effector PKB are involved in growth factor-dependent cell survival (Kauffman-Zeh *et al.*, 1997; Dudek *et al.*, 1997; Kulik *et al.*, 1997 and Yao and Cooper, 1995). Indeed, it was shown previously that IL-13 strongly activates PI 3-kinase as evidenced by PI (3,4,5) P_3 accumulation (Wright *et al.*, 1997). Moreover, data in this study demonstrates that IL-13 also activates PKB and this activation is abrogated by pre-treatment with PI 3-kinase inhibitors. PKB is now known to promote cell survival by phosphorylating a critical serine residue (^{136}Ser) on the death promoting protein Bad, causing it to dissociate from and thus allow activation of the cell survival factor, Bcl-x_L (reveiwed by J. Downward, 1999). However, consistent with observations from other groups (Ossina *et al.*, 1997), Bad is expressed at very low levels in HT-29 cells, such that the band shift of Bad to the serine phosphorylated form was barely detectable. It would seem unlikely, therefore, that the cell survival effects of IL-13 are solely mediated by PKB phosphorylation of Bad in the system described here. However, there are three alternative explanations to account for IL-13-stimulated PI 3-kinase/PKB dependent cell survival mechanisms. First, other death promoting Bcl-2 family proteins may be regulated by PKB-dependent phosphorylation in a manner similar to that described for the regulation of Bad. Indeed, expression of the pro-apoptotic protein Bak, a related Bcl-2 family member, can be directly induced by IFN-γ (Ossina *et al.*, 1997). It would be

interesting to investigate further whether IL-13 can induce hyperphosphorylation of Bak. It remains possible that other Bcl-2 family proteins may act as targets for IL-13-activated PKB. Second, an alternative target for the PI 3-kinase-dependent cell survival signals provided by IL-13 may be the transcription factors of the NF-κB family which have been reported to be important in cell survival by regulating unidentified, anti-apoptotic genes (Wu *et al.*, 1998). Recent evidence has identified the inhibitor of-apoptosis (IAP) proteins, c-IAP1 and c-IAP2, as gene targets of NF-κB transcriptional activity (Wang *et al.*, 1998). The c-IAP1 and c-IAP2 proteins specifically inhibit the active forms of caspase-3 and caspase-7 (Wu *et al.*, 1998). In other systems, such as T lymphocytes, activation of NF-κB has been reported to be dependent on p70 S6-kinase (Lai and Tan, 1994). This in turn has been reported to be a target for phosphorylation by either PKB (Burgering and Coffer, 1995) and/or its upstream kinase(s) PDK-1 and the putative PDK-2 (Alessi *et al.*, 1997 and J. Downward, 1998). Hence, one possibility is that the observed cell survival effects of IL-13 involves PI 3-kinase dependent activation of NF-κB transcriptional activity, although this hypothesis does not fit easily with the recent report demonstrating that IL-13 downregulates TNF-α-mediated activation of NF-κB (Manna and Aggarwal, 1998). Third, PKB has been shown to phosphorylate and consequently deactivate the Forkhead transcription factor FKHRL1 (Brunet *et al.*, 1999). In so doing PKB mediates the downregulation of pro-apoptotic gene transcription and this may well occur in this system through IL-13-induced PKB activation.

This study has some preliminary data on the potential activation of p70 S6-kinase. It would appear that IL-13 is able to induce phosphorylation of certain sites in the auto-inhibitory region. This is thought to relieve a conformation inhibition, which allows for the phosphorylation of ^{389}Thr and subsequent phosphorylation of ^{229}Thr (J. Downward, 1998).

Wortmannin abrogated the IL-13-induced phosphorylation sites in the auto-inhibitory domain, suggesting a role for PI 3-kinase in this activity. However, whether this would constitute an active p70 S6-kinase is unknown. It is tempting to imply a role for IL-13 in cell growth and proliferation, but this is, as yet, unproven. However, this result could implicate a self-regulatory event for the IL-13-induced NF-κB inhibition, in that IL-13 mediates both the inhibition and reactivation of NF-κB, which may account for the partial effect on iNOS, chemokines and survival. Alternatively, IL-13-induced p70 S6-kinase phosphorylation might imply the requirement for protein translation in the mediation of IL-13 effects.

It would be tempting to speculate that the unknown protein pulled down with the SHIP-SH2 domain is the IL-13Rα1 chain, which is the same weight (65-70 kDa). This would again imply that IL-13 could both activate PI 3-kinase and mediate its breakdown. However, there are no commercially available antibodies to test this hypothesis, but could constitute further investigations in the future.

In summary, apoptosis of HT-29 epithelial cells observed in response to a combination of cytokines and/or CD95 ligation is not dependent on NO production. In addition, IL-13 can provide a PI 3-kinase-dependent cell survival signal to HT-29 cells which protects against cytokine-driven apoptotic signals (see Fig. 5.12). The mechanism underlying this cell survival effect of IL-13 is unclear and apparently distinct from the known cell survival signals provided by PKB-dependent phosphorylation of Bad. Nevertheless, our observations indicate a potential role for IL-13 in regulating the controlled program of cell death and survival, a process which plays an important role during several stages of normal colonic epithelial cell development and maturation. Hence, dysregulation of cell survival and death

may be important in the pathogenesis of inflammatory bowel disease and carcinogenesis in the large bowel.

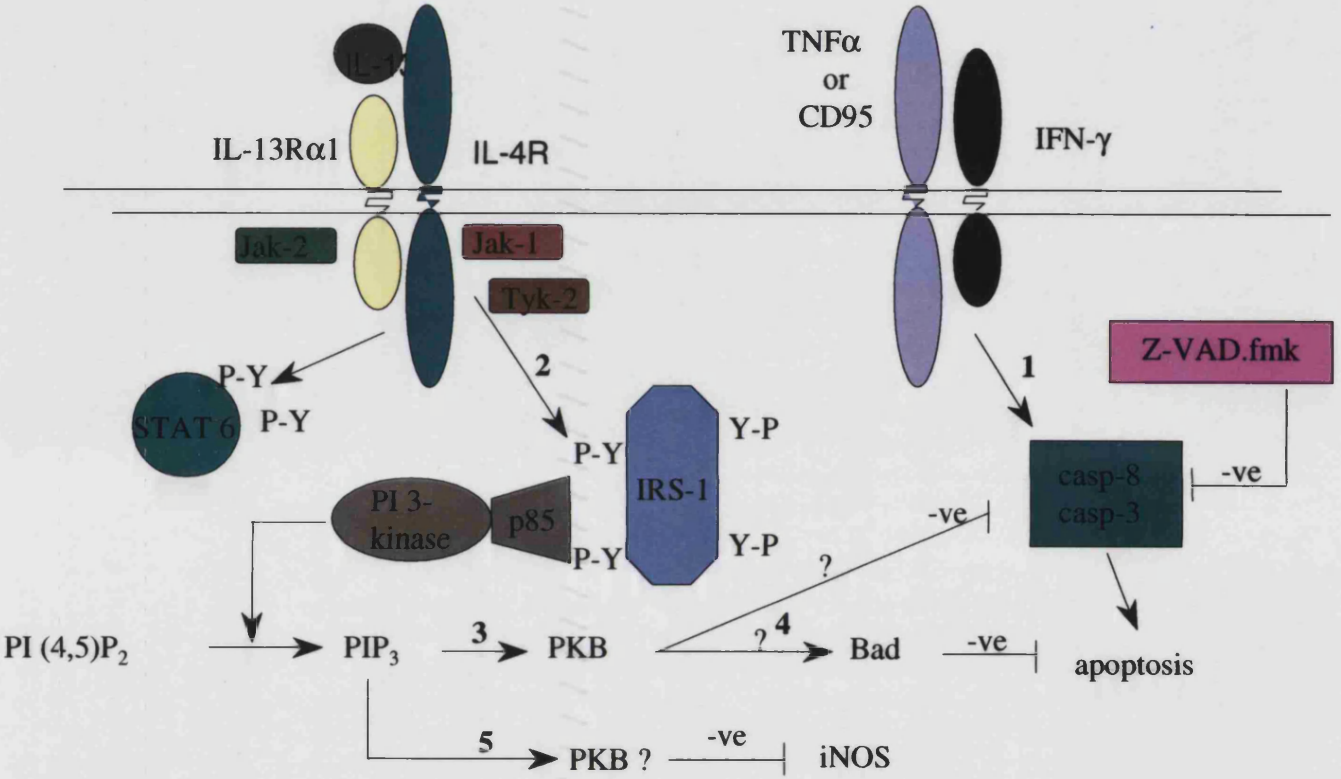


Figure 5.12. Cytokine-induced apoptosis in HT-29 colon epithelial cells. 1. IFN-γ is required in combination with either TNF-α or CD95 ligand in order to induce apoptotic markers, such as caspase activation. 2. IL-13 promotes survival through the activation of PI 3-kinase and its downstream target PKB, 3. The ability of IL-13 to mediate these effects is either direct, 4, through the phosphorylation of proteins which participate in apoptosis, such as the caspases or the Bcl family, or 5, indirect through downregulation of target genes at the transcriptional level. See text for further details.

Understanding how cells integrate signals from a variety of chemically diverse information-containing molecules into complex orchestrated responses such as cell proliferation, differentiation and apoptosis is an extensive goal of cell biology. The ligand molecules that act on the cell surface receptors, including those that mediate proximal aspects of signal transduction, utilise tyrosine phosphorylation events to recruit downstream signalling elements and initiate intracellular signalling pathways. In this study, four different cytokines, namely IL-1α, IFN-γ, TNF-α and IL-13 utilise at least four different pathways to propagate their signals. These include the PI 3-kinase, MAP kinase, JAK-STAT, ceramide and JNK/SAPK pathways. There are some common elements within these pathways. It is possible that a consolidation of signals occurs at various control points such as the adapter molecule, IRS-1 and/or the transcription factor complex, IκB/NF-κB, at which there is modulation of interfering signals and feedback mechanisms. Some of these aspects will be considered in the next section. The phosphorylation status of multifunctional enzymes is likely to affect their activity and for the purposes of this study, the activity of iNOS will be discussed. Interaction between gene products, or cross-talk, may serve as a means to monitor the cell's ongoing response to the many stimuli. An example relevant to this study will also be commented on. Overall, this is an attempt to bring together some of the published work that impinges on the cell model established in this project.

How else could IL-13 regulate apoptosis?

IL-13 may mediate its effects via the IL-4Rα chain. Evidence for this comes from Zamorano *et al*, 1998. They analysed the role that tyrosine-containing domains within the cytoplasmic tail of IL-4Rα play in IL-4-mediated protection from apoptosis. Since the IL-13R in HT-29 cells is comprised of IL-13Rα1 and IL-4Rα chains, some of their findings

may well explain the effects mediated by IL-13 in these cells. IL-4 has been found to regulate apoptosis in a variety of systems (W. E. Paul, 1991; Illera *et al*, 1993; Parry *et al*, 1994; Foote *et al*, 1996; and Dancescu *et al*, 1992). The IL-4Rα cytoplasmic domain contains five tyrosine residues. Y497 is surrounded by a sequence motif (I4R) that is homologous to sequences found in the insulin and insulin-like growth factor (IGF)-1 receptors and is regarded as the growth-promoting domain (see figure 6.1). It appears to be important for IL-4-induced IRS and Shc phosphorylation (Ohara *et al*, 1987 and Wolf *et al*, 1995), cell proliferation and protection from apoptosis (Keegan *et al*, 1994 and Zamorano *et al*, 1996). Y575, Y603 and Y631 comprise the STAT6 docking site, which is regarded as the gene-induction domain (Keegan *et al*, 1994; Wang *et al*, 1996; Pernis *et al*, 1995 and Ryan *et al*, 1996). In addition, the most carboxyl-terminal domain of the huIL-4Rα contains the conserved Y713 surrounded by a proline-rich sequence. Zamorano *et al*, 1998 suggest that the carboxyl-terminal domain can positively regulate IL-4-induced protection from apoptosis and that the STAT6 domain diminished the protection from apoptosis mediated by the I4R domain, in the absence of the carboxyl-terminal domain. Also, their results suggest that IRS-1 is not essential for the carboxyl-terminal-mediated protection from apoptosis, but that Y713 is involved. IL-4Rα was able to phosphorylate SHIP, an ITIM docking site being found on the carboxy-terminal of the IL-4Rα chain (Damen *et al*, 1996), although, Y713 was not essential for this recruitment. They concluded that both Shc and SHIP activation does not correlate with the carboxy-terminal domain's ability to signal protection from apoptosis. Interestingly, they found by site-directed mutagenesis that Y497 and Y713 were able only partially to protect cells from apoptosis, but cooperate to give maximal protection. This finding might bear some relevance to the IL-13-induced inhibition of apoptosis seen in HT-29 cells, where the protection is only partial. Importantly, the ability of the I4R domain to protect from apoptosis seems linked to its

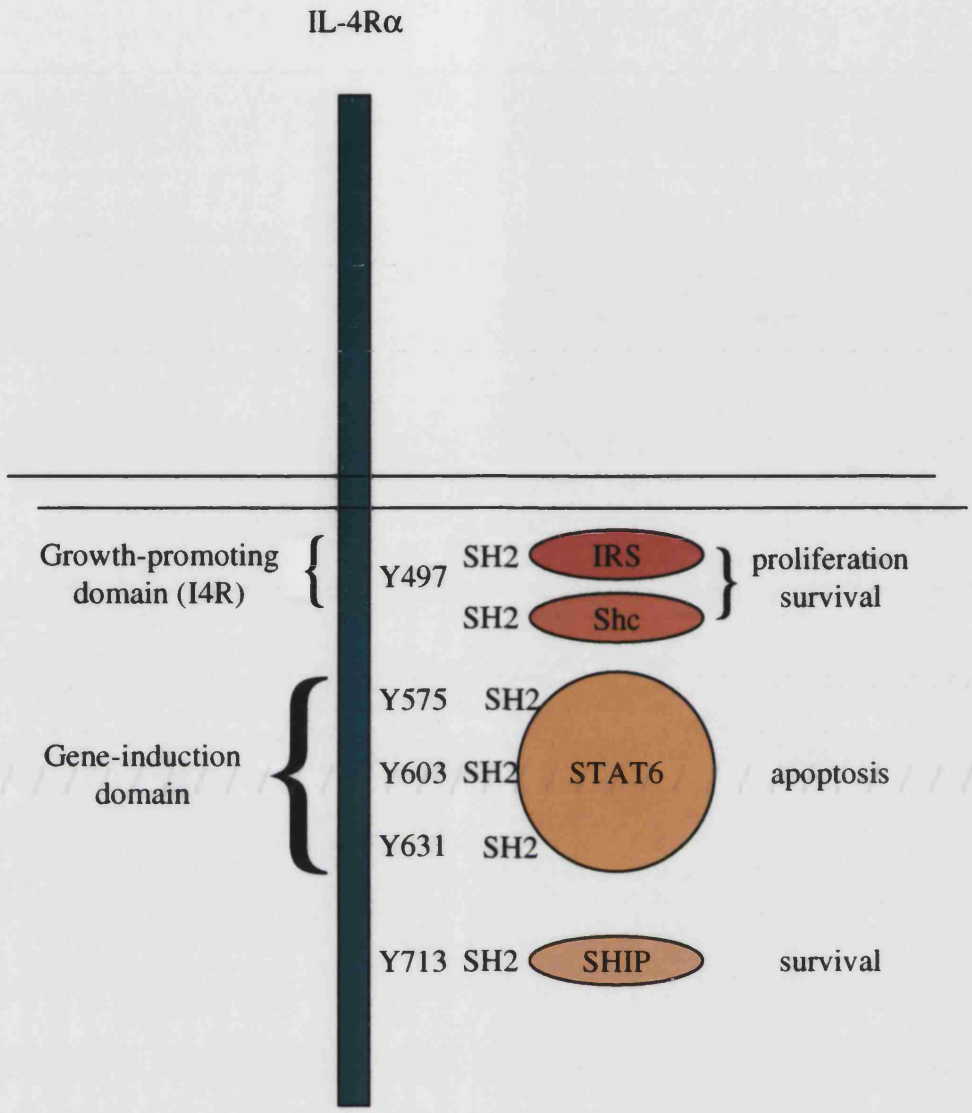


Figure 6.1. Hypothetical model of signalling through the IL-4Rα chain. The cytoplasmic tail of the IL-4Rα chain has five tyrosine residues. These residues are involved in the recruitment of intracellular adapter proteins and the activation of the transcription factors, such as STAT6. These signals have roles in cell proliferation and apoptosis. See text for details. Adapted from Zamorano *et al.*, 1998.

ability to activate IRS-1, whereas the protection mediated by the carboxyl-terminal domain is not related to the induction of IRS-1 phosphorylation. Alternatively, it might be that the STAT6 domain is activating a pathway that negatively regulates protection from apoptosis and the balance of signals promoted by each domain determines the outcome. Put another way, STAT6 could negatively modulate the protective signal mediated by the other domains or block the protective effect when one of the positive signals is off. However, it might be possible that a novel protein involved in anti-apoptotic signals could require both the I4R and the carboxyl-terminal domains to dock to the receptor and perhaps bridge Y497 and Y713 via a dual docking site (Zamorano *et al*, 1998).

Serine/threonine phosphorylation of IRS-1 has been implicated as a negative regulator of insulin signalling (Tanti *et al*, 1994) and TNF-α increases serine phosphorylation through the inhibition of serine phosphatases or activation of serine kinases (Kanety *et al*, 1995). This increased serine phosphorylation interferes with the tyrosine phosphorylation of IRS-1 and also its association with PI 3-kinase (Ricort *et al*, 1997 and Staubs *et al*, 1998). Interestingly, Li *et al*, 1999 found that this serine phosphorylation was mediated by PKB (as a result of PI 3-kinase activity) through, possibly, mTOR or FRAP (R. T. Abraham, 1998; Scott *et al*, 1998 and Burnett *et al*, 1998), suggesting a feedback inhibition. These findings lend support to the idea that there exist complex interactions between signalling pathways and that multisite phosphorylation of IRS-1 allows for the integration of signals.

Beyond the cytokine receptors, there may be compound modulation of the activities of downstream signalling proteins (that is, cross-talk between signalling cascades), which may be important in determining the final outcome of multiple cytokine stimulation. Considerable attention has recently been focused on the role played by different kinase cascades in the control of apoptosis (Xia *et al.*, 1995). Berra *et al*, 1998 suggest that the

inhibition of ERK, by the inactivation of the atypical PKCs, triggers the caspase system which, in turn, stimulates p38. Atypical PKCs are activated by PI 3-kinase products (Akimoto *et al*, 1996; Herrera-Velit *et al*, 1997; Mendez *et al*, 1997 and Sontag *et al*, 1997), which would favour survival through the ERK pathway. Active PKB can directly affect the caspase cascade by deactivating caspase-9 (Cardone *et al*, 1998), whereas, Widmann *et al*, 1998 suggest that caspases cleave signalling molecules such as PKB and MEKK1, thus rendering them inactive. Thus, a critical balance between the ERK, JNK/SAPK, p38 and PI 3-kinase/PKB pathways determines the death or survival of a cell.

Why isn't NO involved in cell death?

HT-29 cells have mutant p53 and are resistant to apoptosis when challenged with a variety of toxic stimuli (Canman *et al.*, 1992) This may be at least partially due to the cells' inability to up-regulate wild-type p53 and the absence of the resulting consequences (induction of G_1 arrest or apoptosis). Ho *et al.*, 1996 found that NO induced nuclear accumulation of p53 protein in a dose- and time-dependent manner, this process mainly being regulated by post-translational modification. Apoptosis was easily induced by NO in COLO205 cells, which contain a wild-type p53, whereas HT-29 cells were resistant to NO-mediated cell death. This is an interesting point because recent work by Ambs *et al.*, 1999 shows that colon tumours from patients exhibit high NOS2 staining in the tumour-infiltrating mononuclear cells. This data correlated with an increase in the number of p53 mutations as the tumours progressed. Tumour-associated NO production may modify DNA directly (Wink *et al.*, 1996) or it may inhibit repair mechanisms (Graziewicz *et al.*, 1996) and, because NO production also induces accumulation of wild-type p53 (Messmer and Brune, 1996 and Forrester *et al.*, 1996), the resulting growth inhibition can provide an additional strong selection pressure for nonfunctional, mutant p53 (Ambs *et al.*, 1997).

In addition to the effects of NO on the control of the expression of p53 (Agarwal *et al.*, 1998) and proteins of the Bcl-2 family (Messmer *et al.*, 1996; Xie *et al.*, 1997; Melkova *et al.*, 1997 and G. Kroemer, 1997), this molecule regulates cell viability through the release of mitochondrial pro-apoptotic mediators that trigger DNA degradation (Kroemer *et al.*, 1997; C. Richter, 1997 and Hortelano *et al.*, 1997). Regarding this apoptotic mechanism, NO induces a large mitochondrial permeability transition, the release of cytochrome *c* and the activation of the caspase cascade (Boscá and Hortelano, 1999). Bcl-2 and Bcl-x_L can prevent the release of cytochrome *c* to the cytosol (Vander Heiden *et al.*, 1997).

Expression of wild-type p53 in HT-29 cells interestingly stimulated an increase in both Bax and Bcl-x_L protein expression (Merchant *et al.*, 1996) and, although cell cycle progression was halted, there was no loss in the cell population. Presumably, apoptosis depends on other variables, although, there could be two additional reasons why NO-mediated apoptosis in the HT-29 cells is dysfunctional and could explain some of the results in this study:

(i) Without the normal NO-induced wild-type p53 rise, Bax levels would not increase. This pathway would normally favour apoptosis, but may not do so in HT-29 cells. It is also thought that p53 regulates accumulation of Bak (Jones *et al.*, 1998), although it can be upregulated by IFN-γ in HT-29 cells (Ossinna *et al.*, 1997), independently, therefore, of p53. It may be that the survival effect of IL-13 may lie in an ability to inhibit IFN-γ-induced Bak expression.

(ii) If Bcl-x_L levels were to increase as a result of NO-mediated increases of p53, an anti-apoptotic signal would arise, so one could argue that NO should be pro-apoptotic in p53-defective HT-29 cells. However, since this does not appear to happen, there may be

some other factor involved. Interestingly, Bcl-x_L is subject to caspase cleavage, which releases the pro-apoptotic C-terminal fragment Bcl-x_S, thereby converting it from a protective protein to a lethal one (Clem *et al.*, 1998), so perhaps p53-mediated increases in Bcl-x_L could still favour apoptosis in the 'normal' setting and does not in HT-29 cells. Lømo *et al.*, 1997 found that IL-13, in combination with CD40L, augmented the expression of Bcl-x_L and Mcl-1, suggesting this as a possible intracellular mechanism of induced survival in B cells.

(iii) There are p53-independent signalling pathways in which NO mediates apoptosis (Messmer and Brune, 1996) and it is, therefore, theoretically possible that these pathways are dysfunctional in HT-29 cells (see Fig. 6.2).

It is conceivably possible that NO still contributes to cell death in colon epithelial cells. An intriguing side issue is the current view that NO can be generated by NOS-independent pathways, such as human xanthine oxidase (Zhang *et al.*, 1998), bacterial nitrate/nitrite reductases (Roediger *et al.*, 1986) and the reaction between hydrogen peroxide and D- or L-arginine (Nagase *et al.*, 1997). These sources of NO are valid alternatives to iNOS in UC (Zhang *et al.*, 1999). Thus, NO may still mediate cell death in HT-29 cells via functional p53-independent pathways and may, indeed, contribute to some extent to the apoptosis seen in this system, an event that would be undetectable with the use of the NOS inhibitor, aminoguanidine. The use of NO donors might help to clarify this question.

Nitric oxide donors are thought to induce stress signalling via ceramide formation (Huwiler *et al.*, 1999). They propose that chronic up-regulation of ceramide levels is achieved by the NO-mediated activation of shingomyelinases and a concomitant inhibition of ceramidases. More notable, however, was their finding that it was the prolonged and sustained increase

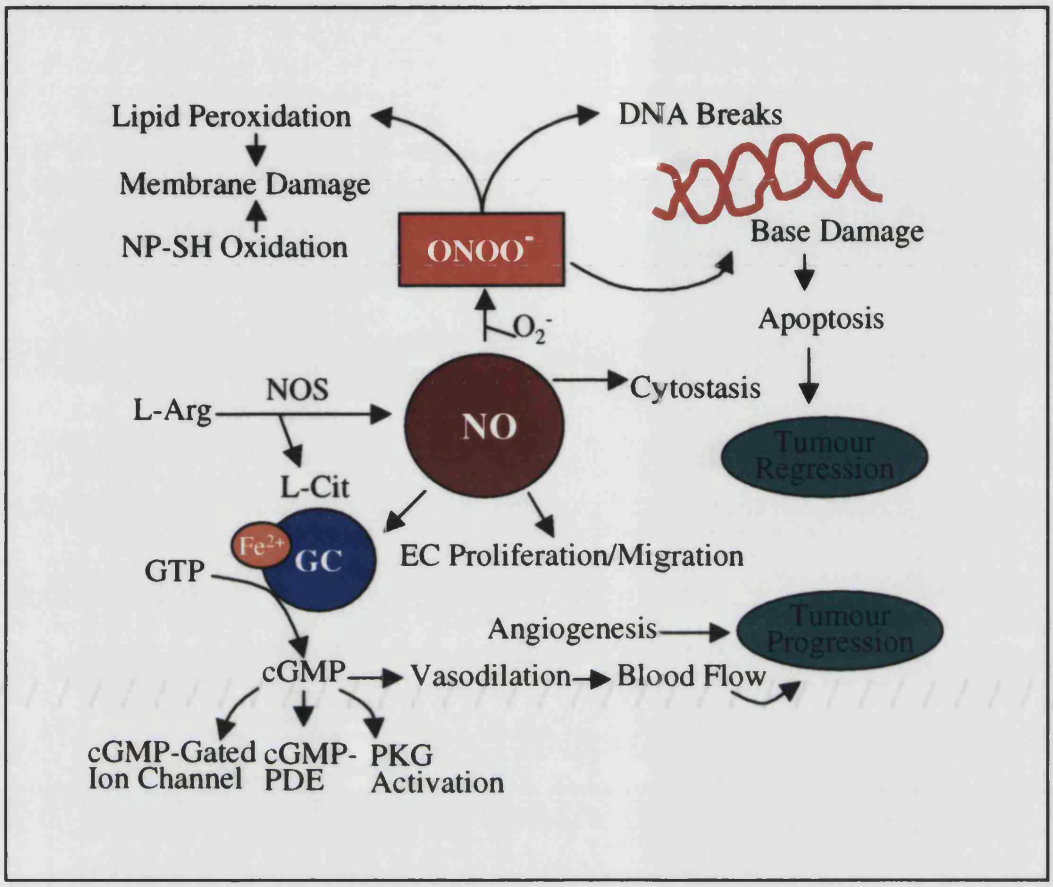


Figure 6.2. Nitric Oxide: A tumour Promoyer or a Tumoricidal Agent? The NO formed by the action of iNOS reacts with oxygen radical and forms highly reactive nitrogen intermediates that serve as endogenous antimicrobial agents. However, excessive production of NO via iNOS, in conjunction with TNF, is also largely involved in IBD. Increased iNOS expression in human cancers indicates that NO has a pathophysiological role in carcinogenesis. The induction of iNOS has also been implicated in increased blood flow and angiogenesis contributing to tumour growth and metastasis. NO is cytotoxic, partly due to its effects on mitochondria. High concentrations of NO can mediate tumoricidal activity, whereas lower concentrations promote tumour growth. On the other hand, generation of high levels of endogenous reactive nitrogen intermediates contribute to induction of apoptosis and inhibition of tumour growth.

of ceramide levels that correlated with apoptosis, unlike TNF-α, which activates both the sphingomyelinases and ceramidases with only transient changes and no net change in chronic ceramide levels. Perhaps this explains why endogenous cytokine-induced NO does not lead to apoptosis, in that there is an internal counterbalance not present with exogenously produced NO (Mühl *et al.*, 1996 and Nitsch *et al.*, 1997).

Phosphorylation of the NOS isoforms is of particular interest, as it would permit cross talk between NO and other signalling pathways and may also serve as a modulator of other post-translational modifications of the NOSs (Michel and Feron, 1997). A new development may have some relevance with regard to the phosphorylation status of iNOS. Activated PKB has now been shown to phosphorylate eNOS and result in NO production (Fulton *et al.*, 1999 and Dimmeler *et al.*, 1999), as well as the demonstration that AMP-activated kinase can phosphorylate human eNOS (Chen *et al.*, 1999). The phosphorylation status of iNOS is largely unknown, although tyrosine phosphorylation has been shown to be important in increasing enzyme activity (Pan *et al.*, 1996). It may well prove to be the case that multisite phosphorylation allows for the integration of signals at iNOS.

Is it all down to NF-κB?

Most of the molecules involved in immune and inflammatory responses are controlled at the transcriptional level by a co-ordinated group of transcription factors. It has been shown that the IκB/NF-κB system is critical in mediating, amongst other things, cytokine-induced iNOS and COX-2 gene expression in intestinal epithelial cells (Jobin *et al.*, 1998).

Jobin *et al.*, 1997 show that HT-29 cells have an altered IκB degradation and incomplete NF-κB nuclear translocation, but that nevertheless, suggest that not only does TRAF-2

play a key role in TNF-α signalling, but that IL-1 also signals through this adapter protein in HT-29 cells (Jobin *et al.*, 1999). Overexpression of IKK in HT-29 cells leads to complete IκB degradation following cytokine stimulation (Didonato *et al.*, 1997) and unpublished work by Jobin *et al* suggests that both IKK activity and IκBα serine 32 phosphorylation are strongly reduced in HT-29 cells. Thus, HT-29 cells have an aberrant cytokine signalling cascade in respect to their IκB/NF-κB axis and this should be taken into account when trying to interpret the outcome of multiple cytokine stimulations.

IL-13 has been shown to inhibit TNF-α-induced NF-κB activation both *in vivo* and *in vitro* (Lentsch *et al.*, 1997 and Manna and Aggarwal, 1998). The mechanism for this is quite complex. IL-13 has no effect on either of the TNF receptors, but rather, mediates its effects by inhibiting IκB degradation (that is, it preserves the protein) and, thus, blocks NF-κB translocation to the nucleus. In addition, IL-13 can block TNF-α-induced MEK kinase (Manna and Aggarwal, 1998), which is a component of the JNK pathway and required for NF-κB activation (Lee *et al.*, 1997). IL-13 was also able to inhibit the activation of NF-κB by a wide variety of agents, including PMA and ceramide (Manna and Aggarwal, 1998). Interestingly, the ability of IL-13 to inhibit NF-κB activation was PKC-, PLC- and PI 3-kinase-dependent. By inhibiting NF-κB activation, IL-13 inhibits NF-κB-dependent gene expression, an important event in both iNOS and COX-2 gene transcription (Jobin *et al.*, 1998).

Besides NF-κB, TNF-α is a potent activator of another transcription factor, AP-1 (Brenner *et al.*, 1989) regulated by the TNF-α-induced activation of JNK (Minden *et al.*, 1994). Manna and Aggarwal, 1998 showed that IL-13 was able to inhibit AP-1 activation by inhibiting JNK activation. In addition, IL-13 was able to inhibit the cytotoxic effects of

TNF-α by the inhibition of caspase-3 in U937 cells. Taken together, it would appear that IL-13 is able to suppress all the effects of TNF-α (Manna and Aggarwal, 1998).

Another intriguing mechanism for IL-13-induced inhibition of NF-κB activation might be the antagonistic role of STAT6. Bennett *et al.*, 1997 found that STAT6 activation by IL-4 blocked TNF-α-induced NF-κB DNA binding in the nucleus, due to overlapping binding sites. They postulate that STAT6 acts as a transcriptional repressor and hypothesise that STAT6 might compete for the IFN-γ-activated transcription factor (GAF) binding sites where there is an overlap. Whether this is a significant mechanism for regulation of all IL-4- and IL-13-responsive genes is unknown at present. Thus, a common theme might be that multisite phosphorylation of the IκB/NF-κB complex allows for an integration of signals.

What has COX-2 got to do with it?

Two cyclooxygenase (COX) isoforms, COX-1 and COX-2, catalyse the synthesis of prostaglandins (O'Neill and Ford-Hutchinson, 1993). COX-1 is expressed constitutively and the prostaglandins produced by COX-1 are thought to play a major role in the maintenance of gastrointestinal homeostasis (T.A. Miller, 1983). COX-2 is induced by pro-inflammatory cytokines IL-1 and TNF-α (Eckman *et al.*, 1997) and the prostaglandins synthesised through COX-2 mediate the inflammatory response (Maier *et al.*, 1990 and Lee *et al.*, 1992). In the normal colonic epithelium there is no significant COX-2 expression, but in ulcerative colitis and Crohn's colitis COX-2 is expressed in epithelial cells in the upper crypts and on the surface but not in the lower crypts (Singer *et al.*, 1998). COX-2 expression may be the result of exposure of cells to pro-inflammatory cytokines (Jung *et al.*, 1995). COX-2 is also expressed in colon cancer and adenomas (Dubois *et al.*, 1996). The mechanism of COX-2 induction in colon cancer is not known, but once

expressed it continues to be expressed and plays a role in carcinogenesis (Oshima *et al.*, 1996). A role for COX-2-derived prostaglandins in colorectal carcinogenesis has been demonstrated using a selective COX-2 inhibitor (Kawamori *et al.*, 1998). These studies on prostaglandins suggest that they play a broad role in regulating epithelial cell proliferation and apoptosis in response to injury and may account for both gastrointestinal tract toxicity of nonsteroidal anti-inflammatory drugs and the role of prostaglandin synthesis in colorectal carcinogenesis (Houchen and Stenson, 1999).

IL-13 down-regulates both iNOS and COX-2 transcription by down-regulation of NF-kB activity (Jobin *et al.*, 1998 and Díaz-Cazorla *et al.*, 1999). This is a potentially confusing picture, in that if COX-2 inhibition results in the abolition of its anti-apoptotic activity (Ho *et al.*, 1998), one could hypothesise that IL-13-induced inhibition would be pro-apoptotic. Due to the multiple anti-inflammatory actions of IL-13, it would not be difficult to presume that multiple controls exist, whereby the many and various ways in which signalling pathways can interact need to be consolidated by the cell before the final outcome can be interpreted. This assertion may account for the partial inhibitory effects of IL-13 on apoptosis because it may be that its anti-inflammatory actions are mediated by the IκB/NF-κB axis counteracted by its anti-apoptotic actions, which are mediated by the PI 3-kinase/PKB pathway.

Finally

In this research project, it has been shown for the first time that the cytokine IL-13 activates the signalling enzyme PI 3-kinase, mediated by the adapter protein IRS-1. In addition, the activation of this enzyme leads to the down-regulation of cytokine-induced iNOS expression and activity. This data was published in 1997 (Wright *et al.*, 1997) and since then there have been a number of publications confirming the role of PI 3-kinase in the regulation of iNOS (Kleinert *et al.*, 1998; Chen *et al.*, 1998; Salh *et al.*, 1998; Pahan *et al.*, 1999; Diaz-Guerra *et al.*, 1999 and Kaliman *et al.*, 1999). Concomitant with this is the ability of IL-13 to delay the onset of cytokine-induced apoptosis, again mediated by the activation of PI 3-kinase and possibly by the activation of its downstream effector, PKB. IL-13 inhibits two crucial effectors of apoptosis, namely caspase-8 and caspase-3, which may constitute the delay seen in cytokine-induced apoptosis. This work has also been published (Wright *et al.*, 1999).

An important clinical arena for the ability of IL-13 to mediate these effects is that of the inflammatory bowel diseases, in that endogenous IL-13 may help to ameliorate some of the symptoms of these diseases. By inhibiting iNOS, the deleterious effects of NO might be ablated and by inhibiting apoptosis, wound healing and the abolition of barrier dysfunction may occur. Interestingly, therapeutic effects have been found for IL-4 gene transfer in experimental inflammatory bowel disease in rats (Hogaboam *et al.*, 1997). However, given the diminished responsiveness of certain elements of the immune system to IL-4 in human IBD patients (Schreiber *et al.*, 1995), it is conceivable that IL-13 gene transfer may circumvent this problem and provide therapeutic potential (Kucharzik *et al.*, 1996). This lends support to the notion that there is therapeutic potential in restoring the balance of cytokines in inflammatory diseases.

Future Directions

Future directions for this work could take many directions:

(i) First, the ability of IL-13 to modulate the activity of NF-κB and activate STAT6 needs to be addressed in the intestinal setting. Establishing whether the PI 3-kinase/PKB pathway modulates these factors, as well as others, such as FKHR, might shed some light on the role of these enzymes in regulating gene transcription. Mimicking these modulatory effects with the use of active mutants of PI 3-kinase and active mutants of PKB, independently of IL-13, with functional readouts including iNOS/chemokine expression and activity, could further clarify the role of this pathway.

(ii) Different IL-13 receptors may have different signalling capacities and this may affect the outcome. Additional work could be carried out in the primary system where the existence of these different receptor complexes is unknown. Culture of primary colonic epithelial cells, although notoriously difficult to do, with further evaluation of the model presented in this project could provide data that would be clinically more relevant.

(iii)It could be clinically beneficial to understand which kinases (for example, PKB) and phosphatases are involved in the post-translational modification of iNOS and whether differential phosphorylation status could modulate the activity of iNOS in different inflammatory settings.

(iv) Using regulated expression of dominant negative versions of class I_A PI 3-kinases would clarify whether the primary role for this class of kinase was proliferation or cell survival. It might also establish a link between PI 3-kinase and caspases in the intestinal

setting. Overall, an investigation into the molecular signalling events which IL-13 uses to mediate its anti-inflammatory activities, may provide further insight into sites for therapeutic intervention.

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